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Improvement in the survivability of *Streptococcus thermophilus* TH982 under simulated gastrointestinal conditions by encapsulation with 2'-fucosyllactose

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“Let food be thy medicine and medicine be thy food”

Hippocrates

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ABSTRACT

Most probiotic strains commercially available today are lactic acid bacteria, which are included in a wide range of product formulations representing a significant sector in the modern functional foods market.

Streptococcus thermophilus is a gram-positive bacterium, widely used in dairy fermentation as starter culture for the production of yogurt and cheese, and it has a potential as probiotic. On the other hand, when ingested it may not survive to passage through the human gastrointestinal tract. Consequently, it is essential to improve cell survivability during gastrointestinal passage, as it is consider a key feature of probiotic strains, and the formulation of these probiotics into microcapsules seems to be a promising emerging solution in addition to controlled release in their target, the gut, to confer health benefit to the host.

The present study is focused on the evaluation of the best encapsulating matrix/agent to fulfil these requirements using an *in vitro* model of human gastrointestinal tract. The molecules used to form sodium alginate based-microcapsules of *S. thermophilus* TH982, were represented by three molecules at 2% (w/v) concentration: a polysaccharide (inulin), a protein (gelatin), and a human milk oligosaccharide (2'-fucosyllactose). Also, capsules of sodium alginate alone were used as control.

The microcapsules were obtained by the extrusion technique, and then the efficiency of the encapsulation process was evaluated, revealing a good efficiency for all the materials to entrap TH982, since more than 90% of total cells were encapsulated.

On the other hand, capsules showed a low decrease in the number of entrapped TH982 after 21 days in skimmed milk at 4 °C, compared to non-encapsulated cells in the same conditions, indicating that it is necessary to entrap a large number of cells to avoid a final count under the recommended least viability (10^7 CFU/g of product at the time of consumption).

Afterwards, the capsules were exposed to 3 h of *in vitro* simulated gastric (pH 2.5) and intestinal (pH 7.5) fluid and the different ability of the encapsulating materials to enhance survival of probiotic strain was compared, also with respect to non-encapsulated TH982.

After stomach and intestine passage, microcapsules were subjected to a simulated intestinal fluid to ensure that the different matrices allowed a release of viable probiotic cells in the intestine.

The oucomes revealed important significant differences among different molecules utilized for the encapsulation of the probiotic strain TH982 of *S. thermophilus*.

RIASSUNTO

La maggior parte dei ceppi probiotici disponibili attualmente in commercio sono i batteri lattici, i quali sono inclusi in un'ampia gamma di formulazioni di prodotti che rappresentano un settore significativo nel moderno mercato degli alimenti funzionali.

Streptococcus thermophilus è un batterio gram-positivo, ampiamente utilizzato nella fermentazione lattiero-casearia come coltura starter per la produzione di yogurt e formaggio, e possiede un potenziale come probiotico. Tuttavia, quando viene ingerito, potrebbe non sopravvivere al passaggio attraverso il tratto gastrointestinale umano. Di conseguenza, è essenziale ottimizzare la sopravvivenza delle cellule durante il passaggio gastrointestinale, poiché è considerata una caratteristica chiave dei ceppi probiotici e l'inclusione di questi probiotici in forma di microcapsule sembra essere una soluzione emergente promettente, oltre al controllo del rilascio nel sito bersaglio, l'intestino, in modo da conferire benefici per la salute dell'ospite.

Il presente studio è focalizzato sulla valutazione della matrice/agente incapsulante in grado di soddisfare questi requisiti utilizzando un modello *in vitro* del tratto gastrointestinale umano. Le molecole utilizzate per formare le microcapsule, che racchiudono *S. thermophilus* TH982, a base di alginato di sodio, sono rappresentate da tre molecole al 2% (w/v) di concentrazione: un polisaccaride (inulina), una proteina (gelatina) e un oligosaccaride del latte umano (2'-fucosillattosio). Inoltre, capsule di alginato di sodio da sole sono state utilizzate come controllo.

Le microcapsule sono state ottenute tramite la tecnica dell'estrusione, e successivamente è stata quindi valutata l'efficienza del processo di incapsulamento, rivelando una buona capacità di intrappolare TH982 per tutti i materiali (più del 90% delle cellule totali è stato incapsulato).

D'altra parte, le capsule hanno mostrato una lieve diminuzione del numero di TH982 intrappolato dopo 21 giorni nel latte scremato a 4 °C, rispetto alle cellule non incapsulate nelle stesse condizioni, indicando che è necessario racchiudere un gran numero di cellule per evitare una popolazione finale al di sotto della minima soglia consigliata (10^7 CFU/g di prodotto al momento del consumo).

Successivamente, le capsule sono state esposte a 3 ore di fluido gastrico (pH 2,5) e intestinale (pH 7,5) simulato *in vitro* e la diversa capacità di migliorare la sopravvivenza del ceppo probiotico è stata confrontata tra i materiali usati per le capsule, e in relazione con TH982 non incapsulato.

Dopo la riproduzione del passaggio nello stomaco e nell'intestino, le microcapsule sono state sottoposte a un fluido intestinale simulato per garantire che le diverse matrici consentissero il rilascio di cellule probiotiche vitali nell'intestino.

I risulati hanno rivelato importanti differenze significative tra le diverse molecole utilizzate per l'incapsulamento del ceppo probiotico TH982 di *S. thermophilus*.

1 INTRODUCTION

The concept of "functional foods" was introduced a long time ago with Hippocrates's motto, "Let food be thy medicine." Recently, evidences started to support the hypothesis that diet may play an important role in modulation of important physiological functions in the body (Vasiljevic and Shah, 2008).

The term probiotic is a relatively new word derived from the Greek *pro bios,* which means for life (Shortt, 1999). The word "probiotic" was initially used as an antonym of the word "antibiotic".

"The history of probiotics began with the history of man" (Soccol et al., 2010). Cheese and fermented milk were well known to the Greeks and Romans, and they suggested their administration for treating gastroenteritis, especially in children. Dairy products are also mentioned in the Bible and the sacred books of Hinduism (Soccol et al., 2010).

The fermentation of dairy products was widely recognized and appreciated early, since olden times, as observed in some products used by the Pharaonic civilization, such as milk, seeds, fish, and some other products (Soccol et al., 2014). It was recognized as one of the oldest methods of food preservation. In the late 19th century, scientists realized that a wide range of traditional sour milk products had auxiliary therapeutical benefits in addition to prolonged shelf-life and pleasant sensory properties (Vasiljevic and Shah, 2008).

1.1 Definition of probiotics

Probiotics have been defined in several ways, depending on our understanding of the mechanisms of action of their effects on humans health and well-being (Salminen et al., 1999).

At the beginning of the 20th century, the main functions of gut flora were completely unknown.

Ilya Ilyich Metchnikoff, the Nobel prize winner in Medicine in 1908, was the first who spotted the effect of what is called today probiotic. He noticed that Balkans (Bulgarian) peasants had an average life-perspective of 87 years, which was not common for the early 1900s. The major difference in comparison with the contemporary diet was large ingestion of bacteria (*L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*) present in yogurt. In "The Prolongation of Life", published in 1907, he postulated that "the dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and replace the harmful microbes by useful microbes." This definition created one of the original hypothesis of the positive role played by certain bacteria: their consumption would alter the composition of the intestinal microbiota towards a gut microbiota that would increase the host health (Metchnikoff, 1907).

From Metchnikoff till today, the probiotic concept has drastically evolved, and different definitions of probiotics have been proposed.

For example, in 1953, the word "probiotika" was used by Werner Kollath, who used it to describe the recovery of malnourished patients by different organic and inorganic supplements (Kollath, 1953).

A year later, Ferdinand Vergin proposed that probiotics were the opposite of antibiotics, and probiotic-rich diet could have restored the microbial imbalance in the body caused by antibiotic treatment. This was considered by many as the first reference to probiotics as they are defined nowadays (Vergin, 1954).

Later on, Lilly and Stillwell (1965), after observations of secretions of *Colpidium campylum* that could increase the growth of *Tetrahymena pyriformis*, defined probiotics as "the secretions of one microorganism which stimulate the growth of another microorganism" (Lilly and Stillwell, 1965).

A powerful evolution of this definition was offered by Parker in 1974, who proposed that probiotics are "organisms and substances which contribute to intestinal microbial balance" (Parker, 1974). Many authors disputed this definition since even antibiotics might have been included (Vasiljevic and Shah, 2008).

In 1989 Fuller attempted to improve Parker's definition of probiotic with the following distinction: "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller, 1989). This revised definition emphasizes the requirement of viable cells as an essential component of an effective probiotic, excludes antibiotics, removing the confusion created by the use of the word "substances", and introduces the concept of a beneficial effect on the host, which was, according to his definition, an animal. However, his definition was more applicable to animals than to humans.

This explanation of probiotic was not wide enough to cover the entire indigenous human microflora. In 1992, another definition was proposed by Havenaar and Huis In't Veld: "probiotics are the viable mono- or mixed microbial cultures which when applied to animal or man have beneficial effects on their host by improving the properties of indigenous microflora" (Havenaar and Huis In't Veld, 1992). In this view, they included the possibility of application to microbial communities at other sites, e.g., respiratory tract, urogenital tract, skin, and not only to the intestinal microflora.

Similarly, in 1996, Salminen broadened the definition of probiotics, defining them as "a live microbial culture or cultured dairy product that beneficially influences the host's health and nutrition" (Salminen, 1996).

A more recent, but not the last definition is "live microorganisms, which when consumed in adequate amounts, confer a health effect on the host" (Guarner and Schaafsma, 1998).

Although all cited authors agreed that probiotics include live microorganisms, Salminen et al. (1999) proposed their view incorporating non-viable bacteria in the following definition: "probiotics are microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host." This definition implies that probiotics do not necessarily need to be viable. Indeed Ouwehand and Salminen (1998) found that non-viable forms of probiotics showed health effects. Furthermore, the definition does not restrict the use of probiotics in foods, and not only whole microbial cells, but also parts of cells have been observed to improve host health (Salminen et al., 1999).

A number of definitions of the term "probiotic" has been used over the years. Currently, the Food and Agriculture Organization (FAO) of the United Nations and World Health Organization (WHO) working group best exemplified the breadth and scope of probiotics as they are known today: "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host", and this definition was reconfirmed by the FAO in 2006 (FAO/WHO, 2006, 2002).

In 2014, this definition was revised with a more grammatically correct elucidation by an expert panel of the International Scientific Association for Probiotics and Prebiotics (ISAPP) as, "live microorganisms that, when administrated in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). This definition includes a broad range of microbes and applications, whilst defining the essence of probiotics (microbial, live, and beneficial to health). However, the use of the word "live" has been controverted since positive effects were also reported for the dead cells (Kareb and Aïder, 2019).

Unfortunately, misuse of the term probiotic has become a significant issue, with many products exploiting the term without meeting the requisite criteria.

1.2 Probiotic microorganisms

Despite these numerous theoretical definitions, however, the practical question arises whether a given microorganism can be considered to be a probiotic or not. Havenaar and Huis In't Veld (1992) proposed some strict criteria for designating a strain as a probiotic: total safety for the host, human origin, survival to gastric acidity and pancreatic secretions, adhesion to epithelial cells, immune modulator activity, production of antimicrobial substances, inhibition of pathogenic bacteria, resistance to antibiotics, tolerance to food additives, and stability in the food (Iacono et al., 2011; Reid et al., 2003).

Although numerous criteria have been recognized and suggested, the probiotics in use today have not been selected according to all of them.

In the last 20 years research in the probiotic area has progressed considerably, and significant advances have been made in selecting and characterizing specific probiotic cultures and substantiating health claims relating to their consumption (FAO/WHO, 2006).

In a recent paper, Morelli (2007) pointed out how approaches in probiotic bacteria selection have been evolved. Several researchers agreed that probiotic strain should be assessed according to the following principle:

- it must be of human origin;
- it must survive during gastric transit;
- it has to tolerate bile salts;
- it has to adhere to gut epithelial tissue.

Based on ecological considerations, these criteria have been used to select strains that have been successfully tested *in vivo* and are currently used as probiotics.

More recently, two additional approaches in probiotic bacteria selection have been suggested by FAO/WHO (2006): the *in vitro* assessment to predict microorganisms functionality in the intestine and genomics analysis. The first is related to the activities exerted by probiotics towards what is termed gut-associated lymphoid tissue (GALT), while the second is the outcome of the availability of the complete genomic sequences of the probiotic strain. There is a large scientific consensus in order to assess the properties of probiotic bacterial strains: it is mandatory to perform a preliminary *in vitro* assessment (Morelli, 2007).

General agreement with regard to potential selection criteria proposed by FAO/WHO (2002) and confirmed by many authors (Holzapfel et al., 1998; Vasiljevic and Shah, 2008) are listed in Table 1.2.

An important operational definition of the term probiotic requires the organism in question to be "consumed in adequate amounts" to confer a benefit. The viability and stability of probiotics have been both a marketing and technological challenge for industrial producers. Probiotic foods should contain specific probiotic strains and maintain a suitable level of viable cells during the product's shelf-life (Mattila-Sandholm et al., 2002). The standard for any food sold with health claims from the addition of probiotics is that it must contain per gram at least 10^6-10^7 CFU of viable probiotic bacteria (FAO/WHO, 2002).

The safety of a strain is defined by the absence of association with pathogenicity and the antibiotic resistance profile (EFSA, 2005). Probiotics are subject to regulations in the general food law, according to which they should be safe for human and animal health. In the USA, microorganisms used for consumption purposes should have the GRAS (Generally Recognized As Safe) status, regulated by the FDA (Food and Drug Administration). In Europe, EFSA introduced the term QPS

(Qualified Presumption of Safety). The QPS concept involves some additional criteria of the safety assessment of bacterial supplements, including the history of safe usage and absence of the risk of acquired resistance to antibiotics (EFSA, 2005). Also, to define a strain as probiotic, safety aspects have to include specifications such as origin (i.e., healthy human gastrointestinal tract) (Mattila-Sandholm et al., 2002). However, the report of the Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics FAO/WHO (2002) pointed out that, at the moment, it is very difficult to identify and confirm the original source of a microorganism. Infants are born without bacteria in the intestine, and the origin of the intestinal microflora has not been fully elucidated. It was emphasized that the ability to remain viable at a specific target site and to be effective are more important than the origin of a specific strain and these properties should be verified for each potentially probiotic strain (FAO/WHO, 2006).

Functional aspects define their survival in the gastrointestinal tract and their immunomodulatory effect. The expected characteristics of probiotic microorganisms may include besides the physiological, immunological and metabolic activity, several technological properties such as been genetically stable strains, have a desired viability during processing and storage, good sensory properties and have a large-scale production (Figueroa-González et al., 2011) (Table 1.2).

It is widely admitted that probiotics must survive through the human gastrointestinal tract (GIT) to exert their beneficial health effects. Therefore, they have to resist to GIT stresses, such as low pH, bile salts and pancreatic secretions. Besides, the dose required for a probiotic effect is still debated, due to fluctuations depending on both the strain and target. Nevertheless, the minimal concentration of cells needed to obtain a clinical effect was quoted to be 10^6 CFU/ml in the small bowel and 10^8 CFU/ml in the colon (Uriot et al., 2017).

Health benefits imparted by probiotic bacteria are strain- and tissue-related and not species- or genus-specific. Thus, the probiotic effect neither universal to all bacterial species nor all human tissues. Moreover, it is important to note that not all the same species' strains will be effective against defined health conditions or to all human tissues (Shah, 2007).

In addition, probiotic effects seem to be dose-dependent (Minelli and Benini, 2008).

The use of probiotics has considerably increased and their potential domain of application in human clinical care is extremely wide: reduction of blood cholesterol, treatment of inflammatory bowel syndrome or gastrointestinal disorders, synthesis of vitamins, protection against antibiotic-associated diarrhea, *Helicobacter pylori* infection, allergic disorders, alleviation of symptoms of lactose intolerance, hypercholesterolemia, respiratory infections and even against systemic disease (Aspri et al., 2020; Kerry et al., 2018; Stavropoulou and Bezirtzoglou, 2020). The clinical utility of probiotics may extend to fields such as allergic disease and cancer. Probiotics have roles in epithelial cell

proliferation and differentiation and the development and the homeostasis of the immune system (Soccol et al., 2014).

With regard to the most studied beneficial health effect, probiotics affect intestinal bacteria by increasing the numbers of beneficial anaerobic bacteria and decreasing the population of potentially pathogenic microorganisms (O'Toole and Cooney, 2008).

In addition, they might influence the intestinal ecosystem by impacting mucosal immune mechanisms, by interacting with commensal or potential pathogenic microbes and by producing metabolic end products such as short-chain fatty acids (SCFAs) (De Melo Pereira et al., 2018). These mechanisms lead to reduction of potential pathogens, an improved intestinal environment, fortification of the intestinal barrier, down-regulation of inflammation, and up-regulation of the immune response to antigenic challenges (Markowiak and Ślizewska, 2017).

These phenomena are thought to mediate the most beneficial effects, including a reduction in the incidence and severity of diarrhea, which is one of the most widely recognized uses of probiotics (Guarner et al., 2017).

Many probiotics currently used come from the human intestine in considerable populations, and commercially the most important strains are lactic acid bacteria (LAB).

LAB play an essential role in food fermentations thus, a wide variety of strains are routinely employed as starter cultures in the industry of dairy, meat and vegetable products (Chow, 2002). The most important contribution of these microorganisms to the product is to preserve the nutritive qualities of the raw material through an extended shelf-life and the inhibition of spoilage and pathogenic bacteria. This is due to competition for nutrients and the presence of inhibitors produced by the starter, including organic acids, hydrogen peroxide, and bacteriocins (O'Sullivan et al., 2002).

Members of the LAB are usually subdivided into two distinct groups based on their carbohydrate metabolism. The homofermentative group, consisting of *Lactococcus*, *Pediococcus*, *Enterococcus*, *Streptococcus* and some lactobacilli, utilizes the Embden-Meyerhof-Parnas (glycolytic) pathway to transform a carbon source primarily into lactic acid.

As opposite, the heterofermentative bacteria produce equimolar amounts of lactate, CO_2, ethanol or acetate from glucose exploiting phospho-ketolase pathway. Members of this group include *Leuconostoc*, *Weissella* and some lactobacilli (Klein et al., 1998).

Lactobacillus and *Bifidobacterium* genera are most commonly used as probiotics, which are considered to be safe based on their historical presence in the human gut and foods.

Other less common species belonging to the strains of *Streptococcus*, *Saccharomyces*, *Escherichia coli*, *Lactococcus*, and *Enterococcus* are also used as probiotics due to their safety and great health potential (Table 1.1).

Table 1.1 - Potentially probiotic cultures used as nutraceuticals and/or in fermented milk. Adapted from Champagne (2005); Kumar et al. (2016); Shah (2007).

Genera	Species
Lactobacillus	*acidophilus*
	delbrueckii subsp. *bulgaricus*
	casei
	crispatus
	johnsonii
	lactis
	paracasei
	fermentum
	plantarum
	rhamnosus
	reuteri
	salivarius
Bifidobacterium	*adolescentis*
	bifidum
	breve
	essensis
	infantis
	lactis
	longum
Enterococcus	*faecalis*
	faecium
Pediococcus	*acidilactici*
Propionibacterium	*freudenreichii*
Saccharomyces	*boulardii*
	cerevisiae
Lactococcus	*lactis* subsp. *lactis*
	lactis subsp. *cremoris*
Streptococcus	*thermophilus*

Lactobacillus includes different species and the most common are: *L. acidophilus*, *L. rhamnosus*, *L. delbrueckii* subsp. *bulgaricus*, *L. reuteri*, *L. casei*, *L. johnsonii*, *L. pantarum*. Lactobacilli are ubiquitous in nature, found in carbohydrate-rich environments. They are gram-positive, non-spore-forming microorganisms, catalase-negative, appearing as rods or coccobacilli. Growth occurs at as high as 45 °C; however, the optimum growth temperature is between 35-40 °C. The organisms grow in slightly acidic media at a pH of 6.4-4.5, but growth ceases when a pH of 4.0-3.6 is reached. The majority of the strains are acid-tolerant in the stomach acidity and have a good adherence capacity to the intestinal cells.

Bifidobacterium belong to the phylum of *Actinobacteria* as they have a characteristic ramified morphology. These bacteria are rod-shaped, non-gas producing and anaerobic organisms. They are

generally characterized as gram-positive, non-spore forming, non-motile and catalase-negative. The most common *Bifidobacterium* probiotic species are *B. animalis*, *B. bifidum*, *B. breve*, *B. infantis*, *B. lactis*, *B. longum*. Optimum growth occurs at a temperature of 37-41 °C and the optimum pH is 6.0-7.0. *Bifidobacterium* are normal inhabitants of the human gastrointestinal tract and selected strains can survive the stomach and intestinal transit and reach the colon in abundant numbers (Shah, 2007); however, the number of bifidobacteria decreases with increasing the age of the host.

Table 1.2 - Key and desirable criteria for the selection of probiotics strains. Adapted from Markowiak and Ślizewska (2017); Vasiljevic and Shah (2008); Morelli (2007).

Criterion	Required property
Safety	Human or animal origin No adverse effects History of safe use Absence of data regarding an association with infective disease Absence of genes responsible for antibiotic resistance localized in non-stable elements
Functionality	Tolerance to gastric acid and juices Bile tolerance Adhesion to mucosal surface Resistance to bacteriocins and acids produced by the endogenic intestinal microbiota Validated and documented health effects Antagonistic activity towards pathogens (e.g., *H. pylori*, *Salmonella* spp., *Listeria monocytogenes*, *Clostridium difficile*) Competitiveness with respect to microbial species inhabiting the intestinal ecosystem
Technological	Guarantee of desired sensory properties of finished products Genetic stability Resistance to bacteriophages Large-scale production Desired viability during processing and storage

Probiotic bacteria are included in a wide range of products, including yoghurts, drinks, capsules, and dietary supplements, and they represent a significant element in the modern functional foods market. The use of diet to fortify certain gut flora components is a popular current aspect of functional food sciences. In this context prebiotics, synbiotics and postbiotics all have a significant role.

1.2.1 Prebiotics

In contrast to probiotics, which are viable organisms, prebiotics describe non-viable food components (Geurts et al., 2014).

In 1995, prebiotics were described by Gibson and Roberfroid as “non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited

number of bacterial species already established in the colon, that can improve host health" (Gibson and Roberfroid, 1995). In 2004, the definition was updated, and prebiotics were defined as a "selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host wellbeing and health" (Gibson et al., 2004). Finally, in 2007, FAO/WHO experts described prebiotics as a "nonviable food component that confers a health benefit on the host associated with modulation of the microbiota" (Chen et al., 2005; Pineiro et al., 2008).

In 1999, Fooks et al. selected some principles which allow the classification of food ingredients as a prebiotic, confirmed in 2009 by Wang:

- they must be not digested (or just partially digested), hydrolyzed or adsorbed in the upper part of the gastrointestinal tract;
- as a consequence, they reach the colon, where they are selectively fermented by potentially beneficial bacteria;
- the fermentation leads to increased production in the relative abundance of different SCFAs, a moderate reduction of colonic pH, reduction of nitrous end products and an improvement of the immunological system (Crittenden and Payne, 2008), which is beneficial for the host;
- selective stimulation of growth and/or activity of the colonic microbiota potentially associated with health protection and wellbeing;
- preferably, prebiotics must be able to endure food processing conditions and remained unchanged, non-degraded, or chemically unaltered and available for bacterial metabolism in the intestine.

Any foodstuff that is not digested by host enzymes and reaches the colon in a practically unaltered form (e.g., non-digestible carbohydrates, some peptides and proteins, as well as specific lipids) where they are fermented by saccharolytic bacteria (e.g., *Bifidobacterium* genus), is a candidate prebiotic.

There are many reports on the beneficial effects of prebiotics on human health. Different studies have suggested that prebiotics affect intestinal bacteria by increasing beneficial anaerobic population, corresponding to stimulation of bifidobacteria and lactobacilli growth, thus conferring important changes in gut microbiota composition and decreasing potentially pathogenic microorganisms (Guarner et al., 2017; Hill et al., 2014).

In addition, further health benefits promoted by prebiotics are: reducing the prevalence and duration of diarrhea, providing relief from inflammation and other symptoms associated with intestinal bowel disorders, and exerting protective effects to prevent colon cancer. Prebiotics are also implicated in enhancing the bioavailability and uptake of minerals, lowering some risk factors for cardiovascular disease, and promoting satiety and weight loss (Kerry et al., 2018).

However, it should be considered that an overdose of prebiotics may lead to flatulence and diarrhea. This effect is absent in the case of excessive consumption of probiotics, in fact they can be consumed on a long-term basis and for prophylactic purposes (Markowiak and Ślizewska, 2018).

There are evidence that the consumption of diets rich in prebiotics is associated with reductions in food intake, body fat content, and body weight gain, especially in overweight and obese individuals (Roberfroid et al., 2010). Dewulf et al. (2013) observe that consumption of prebiotics, compared to a placebo, in obese women, showed complex modulation of the beneficial gut microbial cells after prebiotic treatment: an increase in bifidobacteria is the most common signature of prebiotic treatment with inulin-type fructans.

Furthermore, Bielecka et al. (2002) have shown the relevance of combining prebiotics and probiotics, demonstrating greater effectiveness than probiotics alone.

Prebiotics are naturally occurring in human and animal diets, but they may also be added to food. They can be obtained naturally from sources like vegetables, fruits, and grains consumed in our daily life. Those substances may serve as a medium for probiotics: prebiotics stimulate their growth and contain no microorganisms (Kerry et al., 2018).

Natural sources of potential prebiotics are: tomatoes, artichokes, bananas, starchy fruits, asparagus, berries, garlic, onions, chicory, green vegetables, legumes, beans, as well as oats, linseed, barley, and wheat (Crittenden and Payne, 2008; Markowiak and Ślizewska, 2017). Habitually they are used as food ingredients in, for example, biscuits, cereals, chocolate, spreads, and dairy products (Markowiak and Ślizewska, 2018).

In particular, many food oligosaccharides (between ~2 and 20 saccharide units) and polysaccharides (including dietary fiber) have been claimed to have prebiotic activity, but not all dietary carbohydrates are prebiotics (Kolida and Gibson, 2007). There is accumulating evidence, by *in vitro* and *in vivo* studies, that the most commonly known prebiotics are: inulin-type fructans (ITF), galactooligosaccharides (GOS), fructooligosaccharides (FOS), isomaltooligosaccharides (IMO), xylooligosaccharides (XOS), transgalactooligosaccharides (TOS), soybean oligosaccharides (SBOS), and lactulose (Guarner et al., 2017).

Also, polysaccharides such as cellulose, hemicellulose, or pectin may potentially be prebiotics (Patterson and Burkholder, 2003). Similarly, the use of lactitol, stachyose, raffinose, and saccharose as prebiotics requires further studies (Vallianou et al., 2020) .

Carbohydrates, such as dietary fiber, are potential prebiotics. Prebiotic and dietary fiber are terms used alternatively for food components that are not digested in the gastrointestinal tract; however, there is a significant difference between those two terms: prebiotics are fermented by strictly defined

groups of bacteria, while dietary fibers are used by the majority of colonic microorganisms (Ouwehand et al., 2005).

Prebiotics have enormous potential for modifying the gut microbiota, but these modulations occur at the level of individual strains and species, and thus they cannot be proved theoretically.

1.2.1.1 Inulin-Type Fructans

Inulin-type fructans (ITF) are the most widespread and researched current prebiotics. ITF are present in significant amounts in several fruits and vegetables (Table 1.3). However, these natural sources contain only trace levels of prebiotics, so functional food developments have considered the approach of removing the active ingredients from such sources and adding them to more frequently consumed products to reach an inulin dose of 5-8 g/d that is sufficient to elicit a positive effect on the gut microbiota (Kolida and Gibson, 2007).

Industrial production of inulin almost exclusively uses chicory roots as raw material that belongs to the *Compositae* family. The manufacturing process for inulin is similar to that of sucrose extracted from sugar beets (Figure 1.1): the chicory roots are harvested, sliced, washed, and inulin is extracted from the root by using a hot water diffusion process, then purified and dried (Armisen and Galatas, 2009). Native chicory inulin is obtained as white powder with fine particles with greater clarity and it is a non-fractionated inulin that always contains 6-10% sugars represented as glucose, fructose, sucrose, and small oligosaccharides (Roberfroid, 2005).

For the extraction of oligofructose, the process is the same, but one more step is added: partial enzymatic hydrolysis after extraction. Inulin is broken down using an endo-inulinase (EC 3.2.1.7) into chain lengths ranging from 2 to 10, with an average DP (i.e., degree of polymerization) of 4 (Niness, 1999). Oligofructose can otherwise be obtained by enzymatic synthesis (transfructosylation) using the fungal enzyme β-fructosidase (EC 3.2.1.7) from *Aspergillus niger*.

By applying specific separation technologies, it is also possible to eliminate the oligomers with DP < 10 to produce a high-molecular-weight (long-chain) inulin-type fructan or inulin HP (Roberfroid, 2007).

ITF and oligofructose are part of our daily diet, and thus they are classified as food ingredient, and they have no E-number (not as additives) in all EU countries. In the USA, a panel of experts has confirmed the GRAS status of chicory inulin and oligofructose (Franck, 2002).

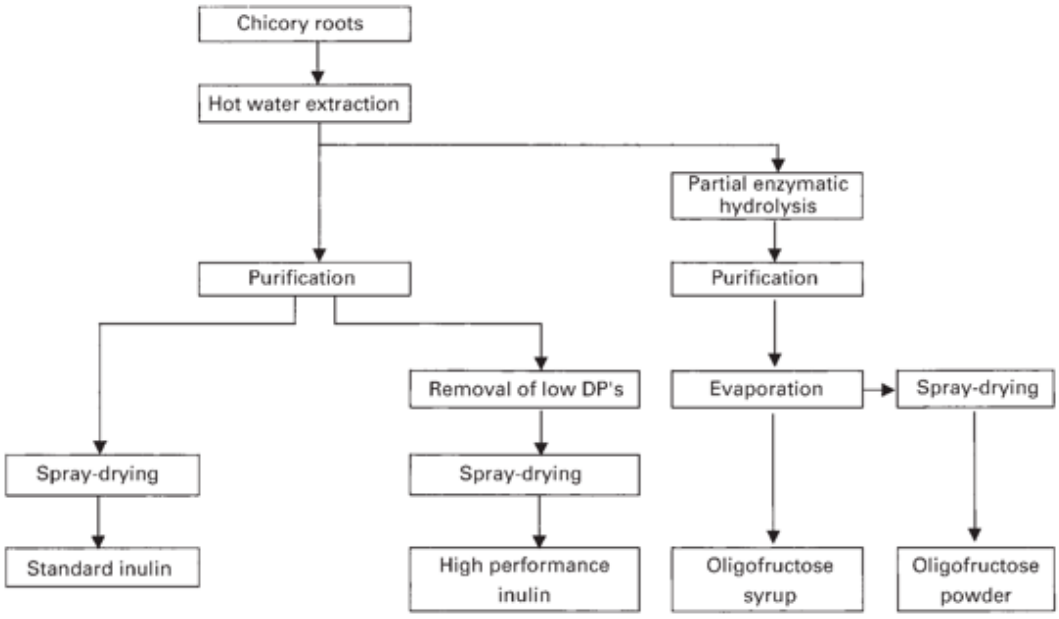


Figure 1.1 - Industrial production process of chicory inulin and oligofructose (Franck, 2002).

DP = degree of polymerization

ITF is the generic term applied to a linear polydisperse mixture of β-2-1 linked D-fructose molecules, otherwise called β-(2,1) fructans. The chain length is a function of the source and moment of harvesting (Armisen and Galatas, 2009) and the DP, generally between 10 and 65. Inulin is built up of 2 ± 60 fructose units with one terminal D-glucose molecule (Figure 1.2) (De Vos et al., 2010).

The term oligofructose or fructo-oligoaccharides, a subgroup of inulin, is used for β-(2,1) fructans with a DP < 10. Indeed, oligofructose and (short-chain) fructo-oligosaccharides are considered to be synonyms to name the mixture of small inulin oligomers (Roberfroid, 2005).

The β-configuration of the anomeric C_2 in the fructose monomers confers to ITF the capacity to resist hydrolysis by human small intestinal digestive enzymes, which are specific for α-glycosidic bonds. They have thus been classified as "non-digestible" oligosaccharides (Roberfroid, 2005). After reaching the colon, they are broken down and utilize through fermentation by bifidobacteria, as they possess the β-fructofuranosidase enzyme, providing a competitive advantage in a mixed culture environment such as the human gut (Kolida and Gibson, 2007). This attitude explains why ITF are classified as dietary fibers.

The prebiotic or bifidogenic effect of inulin and oligofructose has been well established. As described above, ITF are fermented in the large intestine, leading to a stimulation of growth and/or activity of several potentially beneficial intestinal bacteria, specifically bifidobacteria and lactobacilli. This

effect has been observed in children (Kim et al., 2007) and adults (Kolida et al., 2007), with a decrease in the number of potentially harmful species (*Clostridium* spp. and *Bacteroides* spp.).

According to Williams et al. (1994), the administration of the lower dose of 4 g/d of oligosaccharides showed a significant increase in beneficial bacterial groups (bifidobacterial and lactobacilli) count, and *Streptococcus* spp. responded positively to the mixture of oligosaccharides. The cells have been enumerated after recovering from fecal matter.

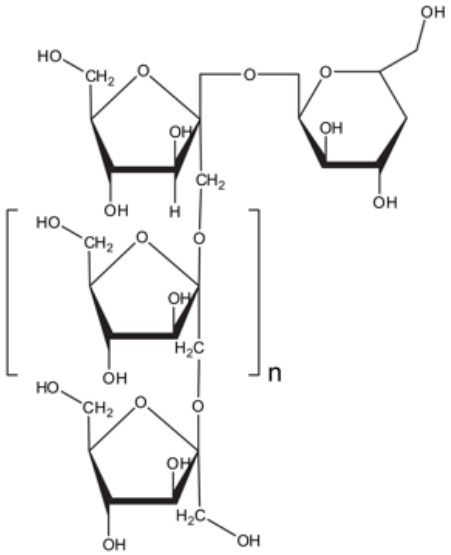


Figure 1.2 - Structure of inulin (De Vos et al., 2010).

The fermentation process products are SCFAs: acetic, propionic, and butyric acid. They can be absorbed and metabolized by the host, and only a third of the caloric content compared with digestible carbohydrates is liberated by this process. This explains why ITF and oligofructose are low caloric food ingredients with a caloric value of about 1.5 kcal/g or 6.3 kJ/g, and thus they are suitable ingredient for diabetics' food (Armisen and Galatas, 2009).

When inulin is fermented by bifidobacteria and SCFAs are produced, it results in a local pH decrease of the large intestine and subsequently an increased in various salts solubility. In fact, calcium available in diet as a mineral or in association with other components should be in ionized form prior to its absorption (Scholz-Ahrens and Schrezenmeir, 2002). Subsequently, the low pH increases the bioavailability of calcium, as well as magnesium and iron (Shoaib et al., 2016).

Additionally, inulin has a notable capacity to replace fat, improving satiety feeling and energy intake. When it is mixed with water or another aqueous liquid, it forms a particle gel network resulting in a creamy white structure, which can easily be incorporated into foods to replace fat by up to 100% (Franck, 2002).

Oligofructose is composed of shorter-chain molecules and possesses functional qualities similar to sugar or glucose syrup. It is useful to partially replace the sucrose molecule's flavor as it provides up to 35% of the sweetness of table sugar, and in addition, it is more soluble than sucrose (Villegas et al., 2010).

The ability to be not digested by small intestinal enzymes qualifies inulin as a matrix molecule for microcapsules because it can reach the colon and survive the upper part of the gastrointestinal tract (De Vos et al., 2010). Moreover, it is cheap, has many health benefits, and can be applied in combination with almost all encapsulation techniques.

1.2.1.2 Human Milk Oligosaccharides

In milk from most species, the prevalent soluble glycan is lactose; in particular, in human milk, lactose is present at concentrations that average 70 g/l (Castanys-Muñoz et al., 2013). It is a digestible disaccharide which provides readily available energy for newborn mammals: in the human small intestine, it is hydrolyzed into its monosaccharide building blocks glucose (Glc) and galactose (Gal), which are then absorbed. In addition, human milk contains a high concentration of diverse soluble oligosaccharides, carbohydrate polymers formed from a small number of monosaccharides (German et al., 2008). The oligosaccharides represent the third-largest substantial component: they can be found in 10 g/l (1%) of mature human milk (Newburg, 2005), and the highest concentration is detected in colostrum (20 g/l) (Coppa et al., 1993). These free soluble milk glycans are known as human milk oligosaccharides (HMOs).

HMOs are synthesized in the mammary gland by specific enzymes, the glycosyltransferases, by adding sequentially one or more monosaccharide units (galactose, fucose, N-acetyl-glucosamine, sialic acid) to the primary acceptor molecule of lactose, thus forming compounds with both linear and branched structures (Coppa et al., 2004). Therefore, lactose is found at the reducing end of all milk oligosaccharides (Castanys-Muñoz et al., 2013).

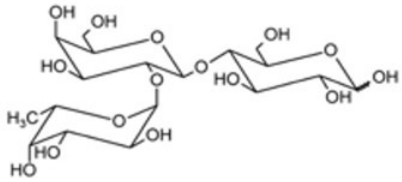
A peculiar characteristic of such substances is that these monosaccharides are linked by specific bonds resistant to the enzymes present in the newborn intestinal wall (lactase, saccharase-isomaltase, maltase-glucoamylase, amylase). As a consequence, most oligosaccharides ingested with mother's milk pass through the small intestine undigested and reach the colon (Coppa et al., 2004). In this regard, the nutritional function most attributed to milk oligosaccharides is to act as soluble prebiotic fiber. There is increasing evidence that HMOs have other functions as well as interaction with immune and epithelial cell receptors, protection of breastfed infants against infections, and inflammation.

The most abundant HMO in human breast milk, which is also available via large-scale commercial production, is the trisaccharide 2'-fucosyllactose (Figure 1.3).

2'-fucosyllactose

One fucosylated substrate of commercial interest for its prebiotic attribute is 2'-fucosyllactose (2'-FL), which has been detected in human milk. 2'-FL can be found in levels of about 2-3 g/l, which counts for approximately 20-25% of total HMOs (Chaturvedi et al., 2001).

An attractive characteristic of 2'-FL is its simplicity, which facilitates its *de novo* synthesis using microbial, enzymatic, or chemical methods (Coulet et al., 2014).



2'-Fucosyllactose
2'-FL
Fuc-α-(1→2)Gal-β-(1→4)-Glc

Figure 1.3 - Structure of 2'-fucosyllactose and possible abbreviation name (Christensen et al., 2020).

This HMO is not present in milk produced by all women, and its concentration varies significantly during lactation and among mothers. This is due to the enzymes necessary for the synthesis of these structures, which are gene products that are not uniformly distributed amongst humans, depending on the genotype of the mother (Thurl et al., 1997). In particular, the synthesis of 2'-FL requires a fucosylation reaction catalyzed by the human α(1→2) fucosyltransferase (E.C.2.4.1.69), or FucT2 (Albermann et al., 2001). An isomer of the 2'-FL present in human milk is 3-fucosyllactose (3-FL) with concentrations of 0.3 to 0.58 g/l in breast milk (Castanys-Muñoz et al., 2013; Christensen et al., 2020).

As above-mentioned, there are variations in the productions of 2'-FL between lactating women due to differential expression of FucT2, which is associated with the same genes that determine Lewis-blood type and Secretor status (Viverge et al., 1990). Other aspects may influence the secretion of 2'-FL, such as general biological variability, ethnicity, and period of lactation (Thurl et al., 2017).

During the last decade, an increasing amount of evidence supporting the potential beneficial effects of 2'-FL as a bioactive molecule has been reported. These properties can be divided into two groups

according to their mechanism of action: a direct effect on the infant by interacting with cells and tissues, and an indirect effect by interacting with beneficial microbiota and pathogens.

The most common causes of infant mortality are infections caused by bacteria and viruses. These pathogens need to attach to specific target cell surface glycans as the first step in pathogenesis, using carbohydrate-binding proteins called adhesins or microbial lectins (Kunz et al., 2000). Fucosylated glycans, such as 2'-FL, may prevent bacterial infections promoting intestinal anti-adhesive function. This is due to the fact that specific HMOs and cell surface glycoproteins and glycolipids (i.e., human cell surface glycans) are synthesized by the same types of glycosyltransferases (Figure 1.4). Hence they would be expected to have structural moieties in common (Chaturvedi et al., 2001; Newburg et al., 2005). In conclusion, 2'-FL from human milk may act as a decoy, inhibiting pathogens' ability to bind to receptors in the gut and thereby protect the breastfed infant from diarrhea (Kunz et al., 2000).

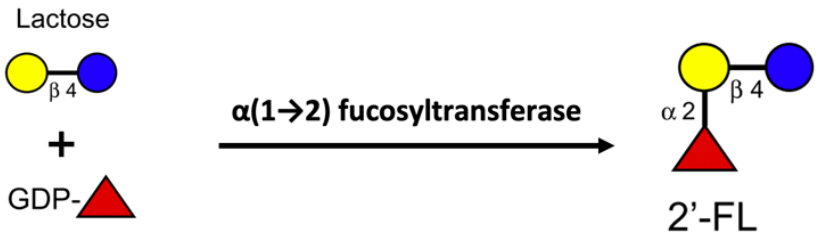


Figure 1.4 - Biosynthesis of fucosylated oligosaccharide 2'-FL. It requires the presence of the sugar nucleotide guanosine diphospho-fucose (GDP-Fuc or GDP- ▲), which acts as a donor of fucose residues (Castanys-Muñoz et al., 2013).

The most common cause of bacterial diarrhea in human is *Campylobacter jejuni* (Ruiz-Palacios et al., 2003). In particular, *C. jejuni* binds specifically to the H-2 epitope (a fucosylated glycan) on human intestinal epithelium's cell surface. Ruiz-Palacios et al. (2003) found that such binding was inhibited *in vitro* by the presence of the milk oligosaccharide 2'-FL exerting competitive binding.

Human milk has also been tested for the ability to inhibit stable toxin (ST) of *E. coli* that causes diarrhea. ST are known to use 2-linked fucosylated glycoconjugates as receptors, as well as *C. jejuni* (Newburg et al., 2004). According to Coppa et al. (2006), ST from enterotoxigenic *E. coli* is inhibited by 2-linked fucosylated oligosaccharides such as 2'-FL. Furthermore, they demonstrated for the first time that human milk oligosaccharides inhibit the adhesion to epithelial cells of aggressive bacteria as *Vibrio cholerae* and *Salmonella fyris*.

In conclusion, they stated that "oligosaccharides are one of the important defensive factors contained in human milk against acute diarrheal infections of breastfed infants" (Coppa et al., 2006).

The prerequisite for prebiotic activity, attributed to 2'-FL, is to resist enzymatic hydrolysis in the gastrointestinal tract. Brand-Miller et al. (1998) provided the first hypothesis that HMOs escape small

intestinal digestion and reach the colon to be fermented by microbiota. Twenty-four healthy breastfed infants have been studied and the breath hydrogen responses to a loading dose of the non-digestible sugar (lactulose) with that after an equivalent load of HMO have been compared. Lactulose is nondigested in the human small intestine but is fermented by bacteria with the production of hydrogen that is easily detected in exhaled air (Engfer et al., 2000). They found that the hydrogen-exhalation profile in infants was not significantly different from that after administration of the equivalent amount of lactulose over 4 hours. In conclusion, this is an indirect proof suggesting that HMOs, of which 2'-FL is the more abundant, resist digestion in the small intestine, and subsequently, they are fermented by bacteria when they reach the colon (Brand-Miller et al., 1998).

This outcome has been further investigated by Engfer et al. (2000), which showed that essentially no hydrolysis occurs in the small intestine, suggesting that the oligosaccharides may reach the colon in an intact form where they serve as substrates for bacterial metabolism, fulfilling the prerequisites required to function as prebiotics. This was supported by the detection with MALDI-MS of the HMOs fraction recovered after digestion, and no hydrolysis products have been identified. Therefore, HMOs might be considered the soluble fiber portion of human breast-milk and thus may stimulate the growth of microorganisms such as bifidobacteria or lactobacilli and produce SCFAs, which facilitate salts and water absorption (Engfer et al., 2000; Gnoth et al., 2000).

HMOs have been detected in the urine of breastfed, but not formula-fed infants, suggesting that they may also act at a systemic level. Since ~1% of the HMOs are absorbed and reach the systemic circulation (Bode, 2009), it is thus probable that they would exert their protective effect in the urinary tract (Martín-Sosa et al., 2002) or modulation of immune responses (Bode, 2012).

1.2.2 Synbiotics

High potential is attributed to the simultaneous use of probiotics and prebiotics (Table 1.3). In 1995, Gibson and Roberfroid, introduced the term "synbiotic" to describe a "combination of synergistically acting probiotics and prebiotics" (Gibson and Roberfroid, 1995). As the word "synbiotic" implies synergy, the term should be used for those products in which a prebiotic component selectively promotes a probiotic microorganism (Cencic and Chingwaru, 2010). An example is fermented dairy products, like yogurt and kefir, which are considered synbiotic because they contain live bacteria (probiotics) and the food source (prebiotics) needed for them to thrive.

The principal purpose of this combination is the improvement of the survival of probiotics in the gastrointestinal tract (Schrezenmeir and de Vrese, 2001). Without its food source, a probiotic would have hurdles surviving in the digestive system because it cannot tolerate low pH and high temperature (Crittenden and Payne, 2008).

A synbiotic product exerts both a prebiotic and probiotic effect, maintaining the balance of the gut flora in healthy individuals and restoring the equilibrium in individuals whose gastrointestinal microbiota has been altered due to illness and/or disease, age, or diet.
Furthermore, *in vitro* studies have shown that synbiotics are more efficient at modulating gut microflora compared to the activity of the probiotics or prebiotics alone (Bengmark, 2005).

1.2.3 Postbiotics

One additional significant term is "postbiotic". Currently, the term "postbiotic", also known as metabiotics, denote "soluble bacterial components with biological activities which are believed to be safer than the use of whole bacteria" (Stavropoulou and Bezirtzoglou, 2020; Tsilingiri et al., 2012).
Recent studies, performed by Patel and Denning (2013), showed that bacterial products, recognized as postbiotics, may have similar effects on signaling pathways and barrier function in the absence of viable organisms. From this view, the following definition was stated: "non-viable bacterial products or metabolic byproducts from probiotic microorganisms that have biologic activity in the host" (Patel and Denning, 2013).
Most common types of postbiotics are metabolic byproducts, such as bacteriocins, organic acids, ethanol, diacetyl, acetaldehydes, hydrogen peroxide, peptides, enzymes, teichoic acids, vitamins, and SCFAs. However, it is also found that certain heat-killed probiotics can also retain important bacterial structures that may exert biological activity in the host (Vallianou et al., 2020) (Table 1.3). SCFAs are metabolites produced by gut microbiota via the fermentation of non-digestible carbohydrates, thus monocarboxylic acids, known for their abundance in plasma and intestines. The most important SCFAs are acetate, propionate, and butyrate (De Vadder et al., 2014). Furthermore, researches showed that these metabolic byproducts have a broad inhibitory property toward pathogenic microbes and, therefore, they can be used as an alternative to antibiotics (Kerry et al., 2018).
In 2009, Mileti et al. (2009) proposed a study to investigate the possible activity of postbiotics, and they detected that a potent postbiotic could protect against the inflammatory properties of invasive *Salmonella* spp. on healthy tissue and also downregulate ongoing inflammatory processes in IBD (inflammatory bowel disease) tissue. "Postbiotics may be a safe alternative to probiotics during the acute phase of IBD" (Mileti et al., 2009).

Table 1.3 - Postbiotics and prebiotics and their natural sources (Kerry et al., 2018).

Bioactive compounds	Natural sources
Postbiotics	
Bacteriocins	*Lactobacillus plantarum* I-UL4
Heat-killed LGG	*Lactobacillus rhamnosus*
Soluble mediator	*Lactobacillus paracasei*
Butyrate	*Faecalibacterium prausnitzii*
Polyphosphate	*Lactobacillus brevis*
Exopolysaccharides	*Lactobacillus pentosus*
Short-chain fatty acids	*Lactobacillus gasser*
Prebiotics	
Fructo-oligosaccharides	Onion, Leek, Asparagus, Chicory, Jerusalem artichoke, Garlic, Wheat, Oat
Inulin	Agave, Banana/Plantain, Burdock Camas, Chicory, Coneflower, Costus, Dandelion, Elecampane, Garlic, Globe artichoke, Jerusalem artichoke, Jicama, Leopard's bane, Mugwort root, Onion, Wild yam
Isomalto-oligosaccharides	Miso, Soy, Sauce, Sake, Honey
Lactulose	Skim milk
Lactosucrose	Milk sugar
Galacto-oligosaccharides	Lentil, Human milk, Chickpea/hummus, Green pea, Lima bean, Kidney
Soybean oligosaccharides	Soybean
Xylo-oligosaccharides	Bamboo shoot, Fruits, Vegetables, Milk, Honey
Fructo-oligosaccharides	Onion, Chicory, Garlic, Asparagus, Banana, Artichoke
Arabinoxylan	Bran of grasses
Arabinoxylan oligosaccharides	Cereals
Resistant starch-1,2,3,4	Beans/legumes, Starchy fruits and vegetables (e.g., bananas), Whole grains

1.3 *Streptococcus thermophilus*

Streptococcus thermophilus is frequently isolated from dairy environments, but recently, Michaylova et al. (2007) reported for the first time isolation and characterization of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* strains from plant samples (*Cornus mas*) in Bulgaria.

S. thermophilus is a gram-positive bacterium widely used in dairy fermentation for the production of yogurt and cheese. It is recognized as anaerobic, aerotolerant, catalase-negative, oxidase-negative, showing ovoid cells occurring in pairs or short chains. It is a thermophilic bacterium with an optimal growth temperature of 42 °C.

S. thermophilus is part of the genus *Streptococcus* (currently known to have more than 70 species), which includes several pathogens (e.g., *S. pneumoniae*, *S. pyogenes*, *S. agalactiae*); however, it has a GRAS status in USA and a QPS status in the European Union, due to a long history of safe use in food production.

Since *S. thermophilus* diverged from its pathogenic relatives, several of the acquired genes appear to originate from other dairy species, such as *Lactococcus lactis* and *L. delbrueckii* subsp. *bulgaricus*, contributing to its adaptation to milk, a well-defined, narrow ecological niche. Moreover it has lost most of the genes responsible for virulence and pathogenic traits (Hols et al., 2005). In particular, genes acquired through horizontal transfer, which encode various functions, such as bacteriocin biosynthesis, lactose and urea utilization, cell-envelope protease and proteins involved in peptide uptake, and exopolysaccharide biosynthesis have been revealed after genomic analysis (Hols et al., 2005). The milk-driven speciation of *S. thermophilus* is also supported by the fact that this species is rarely isolated outside the dairy environment (Martinović et al., 2020).

The genome of *S. thermophilus* is the smallest genomes compared to other LAB and other *Streptococcus* strains (Shi et al., 2015). *S. thermophilus* is related to *L. lactis* but is phylogenetically closer to streptococcal species of the viridans group, which is divided into five categories: *mutans*, *anginosus*, *sanguinus*, *mitis*, and *salivarius*. The last one includes *S. salivarius*, *S. vestibularis* and *S. thermophilus*. *S. salivarius* and *S. vestibularis* are commensal bacteria of the oral and gastrointestinal cavities and genital tract (Kawamura et al., 1995).

The taxonomic status of *S. thermophilus* has been controversial: it was classified as a *S. salivarius* subspecies (*S. salivarius* subsp. *thermophilus*) until Schleifer et al. (1991) through DNA-DNA hybridization studies under stringent conditions and physiological data provided sufficient evidence for conferring species rank on *S. salivarius* subsp. *thermophilus*.

1.3.1 Technological and functional attributes of *S. thermophilus*

S. thermophilus is the second most important species of industrial LAB after *L. lactis*, and one of the basic starter bacteria of yogurt. *S. thermophilus* is the only *Streptococcus* species used in the food industry.

Beyond the common practice in combination with *L. delbrueckii* subsp. *bulgaricus* in yoghurt, *S. thermophilus* is used to produce several varieties of so-called hard “cooked” cheeses (e.g., Swiss cheese, Brick cheese, Parmesan, Provolone, Mozzarella, and Asiago) at a relatively high process temperature (45 °C). In the cheese industry, *S. thermophilus* is mainly used in the refining of hard cheeses through the production of aromatic compounds from amino acids due to its glutamate dehydrogenase activity.

One of the main functions of *S. thermophilus* in yoghurt production is to provide rapid acidification through the conversion of lactose into lactic acid. This aspect is linked to food safety, since most pathogenic bacteria grow very slowly or not at acidic pH. In addition to lactic acid, it also produces secondary fermentation products such as formate, acetoin, diacetyl, acetaldehyde, and acetate, which

contribute to the typical aromatic flavor of fermented products, while the creation of the viscous texture is provided by the production of exopolysaccharides.

Furthermore, *S. thermophilus* has several technological properties, such as sugar metabolism, galactose utilization, proteolytic activity, and urease activity. These diverse technological abilities represent the phenotypic diversity existing within the species (Iyer et al., 2010b).

Therefore, in contrast with other LAB, the probiotic status of *S. thermophilus* remains a point of debate.

1.3.2 Probiotic attributes of *S. thermophilus*

S. thermophilus is well known for several functional and technological attributes such as the production of extracellular polysaccharides, bacteriocins, and biosynthesis of folate. Besides, it also has potential as a probiotic, as demonstrated by various health effects.

S. thermophilus is of nonhuman origin, and although it is known to be sensitive to acidic gastric conditions, it has been shown to survive gastrointestinal transit and moderately adhere to intestinal epithelial cells (Iyer et al., 2010b). Therefore, *S. thermophilus* could be considered as a transient probiotic (Collins et al., 1998; Iyer et al., 2010b).

A large number of *in vivo* studies in humans or animals have also shown beneficial health effects for *S. thermophilus*, such as improving intestinal microflora and lactose digestion in lactose-intolerant individuals (Shah, 2007), stimulating the gut immune system (Delorme, 2008), production of high quantity of folate extracellularly (Tarrah et al., 2018), and prevention of infectious diarrhea (Canani et al., 2007).

Nevertheless, the available data should be interpreted with caution as in most of these studies, *S. thermophilus* was administrated with another LAB strain or within yogurt (Uriot et al., 2017).

Also, it is necessary to take into consideration that the survival and beneficial effects of *S. thermophilus* are widely strain-dependent, as other probiotic microrganisms; hence it is expected that only certain strains could obtain the probiotic status after clinical studies.

1.3.2.1 Alleviation of lactose intolerance

Among the above-mentioned LAB, *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, which are traditionally used in yoghurt production, have not been considered probiotics during a long time by most scientists because some studies reported that they do not survive in the gastrointestinal tract and are not natural inhabitants of the intestine (Senok et al., 2005).

However, in 2010, the European Food Safety Authority (EFSA) has granted a health claim, formulating that “a cause-and-effect relationship has been established between the consumption of

live yoghurt cultures in yoghurt and improved digestion of lactose in yoghurt in individuals with lactose maldigestion" (EFSA, 2010). Individuals with lactose intolerance suffer from excessive flatulence, abdominal pains, and diarrhea due to a deficiency of the enzyme β-galactosidase in the small intestine. When the β-galactosidase is present, lactose is hydrolyzed into its constituent monosaccharides, glucose and galactose, which are transported across the small intestine epithelium. These two cultures contain substantial quantities of β-D-galactosidase (Shah, 2007), and thus yoghurt is well tolerated by individuals with lactose maldigestion.

To bear the claim, yogurt must contain at least 10^8 CFU live microorganisms of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* per gram of fermented product (EFSA, 2010). The claim has been attributed to yogurt containing both bacteria, but not to *S. thermophilus* alone.

Then, even if the beneficial effect of yogurt on alleviation of lactose intolerance is well established by clinical studies, yet no solid evidence in humans has been presented to state that this effect can be specifically attributed to *S. thermophilus* (Uriot et al., 2017). Even if yoghurt properties are approved by EFSA, the probiotic status of *S. thermophilus* is still questioned.

1.3.2.2 Proteolytic system

LAB are nutritionally fastidious, needing an exogenous supply of amino acids to initiate growth (Iyer et al., 2010b).

The proteolytic system of *S. thermophilus* is similar to that found in other LAB. It includes more than 20 proteolytic enzymes, divided into (Hols et al., 2005):

- an extracellular cell-anchored protease capable of casein hydrolysis;
- a set of amino acid and peptide transport systems required for import of amino acids;
- a set of intracellular peptidases involved in the hydrolysis of casein-derived peptides essential for various house-keeping processes.

Most of *S. thermophilus* strains appear to be auxotrophic. They require an exogenous supply of amino acids for growth (at least four amino acids: Glu, Cys, His, and Met), while other LAB are known to be less demanding.

Since milk contains low quantities of free amino acids and short peptides, optimal growth of *S. thermophilus* in milk requires hydrolysis of caseins, internalization and hydrolysis of the resulting peptides, or *de novo* amino acid biosynthesis (Hols et al., 2005). Bacteria used for yoghurt production release higher amounts of free amino acids and have greater aminopeptidase and dipeptidyl-peptidase activity than the others probiotic bacteria. As a result, yogurt bacteria grow faster in milk, whereas other bacteria grow slowly due to low proteolytic activity.

S. thermophilus has also been studied for its ability to hydrolyze the major whey proteins, β-lactoglobulin (BLG) and α-lactalbumin (ALA), increasing the digestibility (El-Zahar et al., 2004). The catabolism of amino acids plays an important role in providing precursors for the biosynthesis of amino acids, nucleotides, vitamins, and the production of a large number of key aroma compounds of yoghurt.

Helinck et al. (2004) tested three LAB species, and revealed that only *S. thermophilus* exhibit GDH (glutamate dehydrogenase) activity that produced α-KG (α-ketoglutarate), an aroma compound from glutamate transamination; however, the intensity of activity was strain-dependent (Helinck et al., 2004).

The characteristic flavour component in yoghurt, acetaldehyde, is also essentially a product of amino acid catabolism. The relatively high concentration of acetaldehyde (in the range of 5 to 21 mg/l) in yogurt could be due to a low utilization rate of this metabolite since *S. thermophilus* lacks alcohol dehydrogenase, the main enzyme for acetaldehyde conversion into ethanol.

In addition, Chaves et al. (2002) suggested that, in the specific case of *S. thermophilus*, threonine can be directly converted into acetaldehyde and glycine by the serine hydroxymethyltransferase (SHMT; EC 2.1.2.1). This is an important enzyme involved not only in the formation of glycine and serine but also in the turnover of folate in all organisms (Chaves et al., 2002).

1.3.2.3 Proto-cooperation

S. thermophilus is used together with *L. delbrueckii* subsp. *bulgaricus* for yogurt making, leading to a complex symbiotic relationship, so called "proto-cooperation" by Hols et al. (2005), sharing the same ecological niche: *S. thermophilus* produces CO_2 and formic acid, which stimulate the growth of *L. delbrueckii* subsp. *bulgaricus* while *L. delbrueckii* subsp. *bulgaricus* hydrolyses milk proteins releasing peptides and amino acids that improve *S. thermophilus* growth (Uriot et al., 2017).

Moreover, Arioli et al. (2017) explained how microbial interactions leading to positive effects are classified as mutualistic interactions. They give positive effects on each population's growth rate and size, while the independent growth of the two species in milk led to a slower growth rate and a smaller population size. In particular, Arioli et al. (2017) considered that among the LAB involved in dairy fermentation processes, urease activity is present only in *S. thermophilus*.

Urea hydrolysis increases the catabolic efficiency of *S. thermophilus* by modulating the intracellular pH and increasing the activity of β-galactosidase, glycolytic enzymes, and lactate dehydrogenase. Simultaneously, urea hydrolysis results in increases in intracellular (pHin) and extracellular (pHout) pH due to the rapid diffusion of ammonia outside of the cell. Consequently, in the presence of urea and a urease-positive microorganism (*S. thermophilus*), urease-negative microorganisms (*L.*

delbrueckii subsp. *bulgaricus*) share the environmental benefit derived from the release of ammonia (local pH increase). To put it briefly, the homolactic fermentation of *L. delbrueckii* subsp. *bulgaricus* appeared to be boosted by ammonia released from urease activity of *S. thermophilus*. Urease production should be considered an altruistic cooperative trait: it is costly for urease-positive individuals but provides a local benefit because other individuals can take advantage of the release of ammonia (Arioli et al., 2017).

1.3.2.4 Bacteriocin production

Antimicrobial activity is one of the main trait of probiotic properties.

Many studies have characterized several bacteriocins produced by *S. thermophilus* showing to have *in vitro* inhibitory activities against LAB but also against gram-positive pathogenic strains such as *E. faecalis*, *Clostridium botulinum*, *Staphylococcus aureus,* and *L. monocytogenes* (Fontaine and Hols, 2008). Some bacteriocin-producing strains could be used in thermophilic starters since not all bacteriocins are active against thermophilic lactobacilli (Delorme, 2008).

Bacteriocins produced by *S. thermophilus* strains are known as thermophilins, small peptides able to inhibit the growth or kill closely related bacteria. Rossi et al. (2013) defined them as "proteinaceous compounds" that are thermostable and active over a wide range of pH values, unlike nisin, which is not used in acidic foods. Moreover, they are safe because of the GRAS status of *S. thermophilus*.

Production of thermophilins represents a significant interest for conservation in the food industry because it may limit the proliferation of pathogenic bacteria in dairy products fermented by *S. thermophilus*. However, as a part of probiotic criteria, it is necessary to test whether these bacteriocins are produced in the GIT and are effective against pathogenic bacteria in such environment.

1.3.2.5 Exopolysaccharide biosynthesis

Extracellular polysaccharides are synthesized by a great variety of bacteria, including LAB, and specifically main strains of *S. thermophilus*. Bacterial exopolysaccharides (EPS) can be composed of one type of sugar monomer (homopolysaccharide) or several types of monomers (heteropolysaccharide). Heteropolysaccharides are synthesized by many LAB, including *S. thermophilus*; indeed, compositional and structural analyses of EPS revealed the presence of galactose, glucose, and rhamnose, as well as N-acetyl-galactosamine, fucose, and acetylated galactose (Iyer et al., 2010b).

These polymers may be assembled as capsular polysaccharides, tightly associated with the cell surface, or they may be liberated into the extracellular environment (i.e., "ropy" polysaccharide).

Although the presence of exopolysaccharide does not confer any direct advantage to growth or survival of *S. thermophilus* in milk, incorporation of EPS or EPS with respective culture in dairy foods can improve rheological properties, such as viscosity, stability, and water-binding. All these characteristics contribute positively to the mouth-feel, texture, and taste perception of fermented dairy products (Angelin and Kavitha, 2020; Rodríguez et al., 2009).

Starter cultures producing EPS may be used to control syneresis in yogurt. This method is particularly widespread in countries where the addition of plant- or animal-derived stabilizers is prohibited or to satisfy modern consumer preferences for products with low content of food additives.

The ability to produce EPS in *S. thermophilus* is both strain-dependent and significantly affected by media and growth conditions (e.g., temperature, carbon: nitrogen ratio, pH). Generally, the amount of EPS produced by *S. thermophilus* in milk may range from 50 mg to 1.5 g/l (Broadbent et al., 2003). In nature, bacterial EPS are thought to fulfill diverse functions that may include cell protection against toxic environments and other antagonisms, sequestering of essential cations, colonization, and in cellular recognition (Broadbent et al., 2003).

Rodríguez et al. (2009) evaluated the potential antigastritis effect of fermented milks with EPS-producing *S. thermophilus* strains using a murine model of chronic gastritis induced by acetyl-salicylic acid (ASA). ASA is a nonsteroidal anti-inflammatory drug used worldwide as an analgesic and antipyretic agent. Furthermore, it is one of the main causes of gastric damage (i.e., gastrointestinal complications ranging from dyspepsia and abdominal pain to gastric ulcers) in people having an excessive consumption of this drug. This study showed the gastro-protective effect of EPS produced by the *S. thermophilus* strain. Fermented milks with EPS-producing strains on gastric mucosa chronic lesions induced by ASA may be used as a safer and natural gastroprotective alternative, since EPS could stimulate the immune system and exert an inhibitory effect on ulcer in the host.

1.3.2.6 Biosynthesis of folate

Folate is an essential component in the human diet involved as a cofactor in many metabolic pathways, such as DNA replication and biosynthesis of nucleotides (Sybesma et al., 2003). Folate is a water-soluble vitamin, and its deficiency in humans is quite widespread. It is associated with several health problems: increased cancer risk in adults and neural tube defects during embryo development (Crittenden et al., 2003). The daily recommended intake for an adult varies from 200 µg in Europe to 400 µg in the United States (Sybesma et al., 2003).

Therefore, an exogenous supply of folic acid appears essential to prevent nutritional deficiency. Recently, on the opposite side, several studies have shown that high intakes of folic acid, the chemically synthesized form of folate, in some cases can cause adverse effects (Iyer et al., 2010a).

For these reasons, many researchers are looking for novel natural sources to increase concentrations of occurring folate variants in foods. Folate is produced by various green plants and by some microorganisms; indeed vegetables and dairy products are the main sources of folate for humans. Milk is a well-known source of folate, and fermented milk products are reported to contain up to 110 µg of folate per liter as a result of the production of additional folate by the LAB in the yogurt (Sybesma et al., 2003).

Numerous researchers have reported that the two LAB species in yogurt, *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*, can synthesize folate, but only the latter is reported to produce high quantities, and the majority of this is excreted into milk (Crittenden et al., 2003; Iyer et al., 2010a).

Tarrah et al (2018), studying eight different strains of newly isolated *S. thermophilus*, showed that all strains increased the amount of folate during growth, concluding that *S. thermophilus* has a strain-specific ability for folate production (Tarrah et al., 2018).

Hence the application of high-folate-producing *S. thermophilus* strains could lead the question of folate deficiency with its natural folate production ability, which can fulfill the daily recommended requirement of folate (200 µg) (Iyer et al., 2010b).

1.3.2.7 Treatment of diarrhea

S. thermophilus has been shown to have positive effects on diarrhea in young children, enterocolitis in premature neonates, and inflammatory gut disease (Delorme, 2008).

Acute diarrhea is a serious cause of infant mortality resulting from viral (rotavirus) or bacterial infections in the gut (Uriot et al., 2017).

In a large study, involving 571 children, Canani et al. (2007) administered five different probiotic preparations, testing their effectiveness in reducing diarrhea duration. The median duration of diarrhea was significantly shorter (70 hours) in children who received the mix of four bacterial strains, including *S. thermophilus*, than in children who received oral rehydration solution alone (115 hours).

Antibiotic-associated diarrhea (AAD) is the most common adverse effect of antibiotic therapy, occurring in 1/3 of patients (Beniwal et al., 2003).

Two major forms of AAD have been identified:

1. No pathogen-related. Pathogenesis may be related to altered SCFAs in the intestine, functional disturbance of bile acid metabolism due to alteration of the microflora.
2. *Clostridium difficile*-associated diarrhea (10-20% of AAD). Pathogenesis may be related to loss of the resistance provided by normal colonic flora. Antibiotics suppress the intestinal microflora that control the proliferation of *C. difficile*.

In a trial conducted by Beniwal et al. (2003), 202 adult patients received 227 g of yogurt per day for 8 days. Results showed that a dietary supplement of yogurt decreased the risk of developing AAD by nearly 50% ($P = 0.04$), and the total number of diarrheal days was 60 in the control group and 23 in the yogurt group.

In conclusion, Beniwal et al. (2003) stated that "during antibiotic therapy, supplementation with commercially available yogurt that contains active cultures is a simple, safe, and cost-effective method of reducing the occurrence and severity of AAD".

1.4 Gastrointestinal tract and its microbiota

Some ecological considerations on the gut flora are necessary to understand the relevance, for human health, of the probiotic food concept.

The human gastrointestinal tract (GIT) is approximately 7 m long, and the gastrointestinal mucosa is the largest surface area within the body (approximately 400 m^2), with only a single layer of epithelial cells separating the contents of the lumen from the internal milieu (Shanahan, 2002).

The development of human microbiota is a dynamic process, beginning with birth. In the uterus, infants grow in a sterile environment. During the first days of life, the intestine becomes inhabited by microbes and is characterized by instability. Subsequently, the microbiota will stabilize during breast or formula feeding. The fecal flora of breastfed infants is dominated by *Bifidobacterium* and *Ruminococcus*, while formula-fed infants have a more complex microbiota with enterobacterial genera, *Streptococcus*, *Bacteroides, Clostridium*, *Bifidobacterium,* and *Atopobium* (Salminen et al., 1998).

The next great changes in the composition of the intestinal microbiota come with the introduction of solid food: a more stable community similar to the adult microbiota becomes established after weaning (at 2-3 years of age) (Power et al., 2014). After this, only relatively small changes take place, and a child has an adult-like microbiota by approximately two years of age.

The microbes that are present in the microbiota have been distinguished into two categories (Zoetendal et al., 2008):

- individual core: continuously present in an individual's microbiota, representing the stable colonizers of the healthy individual. It is determined partly by the host genotype, by initial colonization at birth via vertical transmission, and by dietary habits (Guarner et al., 2017);
- common core: a limited number of microbial phylotypes shared by different people.

During adult life, the stability of the composition of the intestinal microbiota at the species level is relatively common, while stability at the level of bacterial strains may be less common (Mccartney et al., 1996). This relative stability is reduced in old age. Furthermore, the microbiota is then driven

by a series of complex and dynamic interactions. The presence of major stressors, as environmental stresses, medication, extreme based diets, and most noticeably antibiotic treatment may lead to perturbations of the intestinal microbiota.

The intestinal mucosa is said to contain more nerve endings than any other tissue in the body. Severe and prolonged states of stress have been shown to have profound effects on the individual, causing gut-derived sepsis. It is considered as a systemic inflammation caused by the release of norepinephrine into the intestinal lumen, leading to changes in the beneficial bacteria and alterations of the phenotype of the potentially pathogenic microorganisms, which become considerably more virulent and sometimes life-threatening (Alverdy et al., 2003).

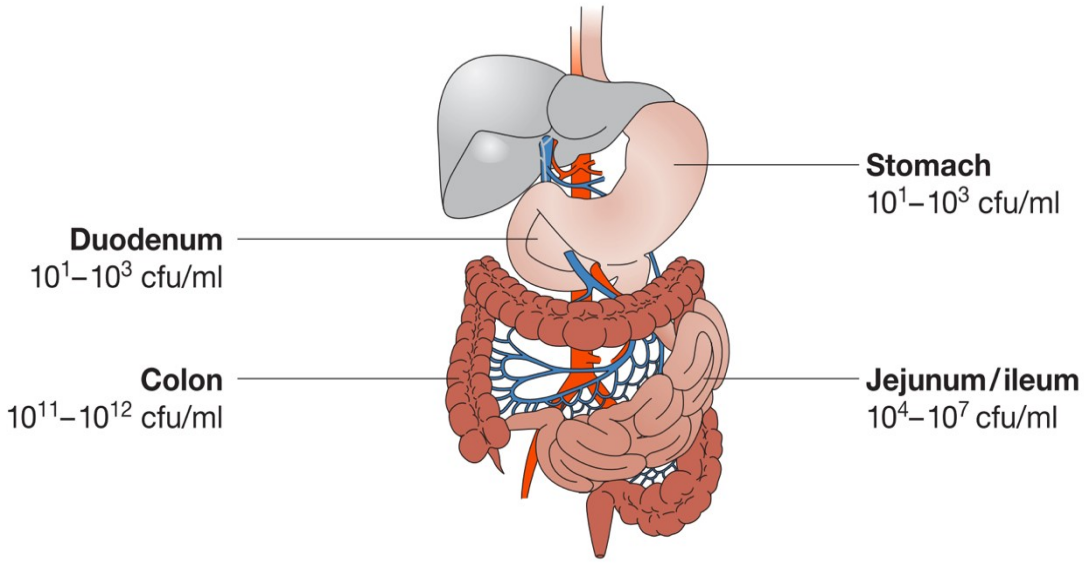


Figure 1.5 - Bacteria density increases in the jejunum and in the ileum from the stomach and duodenum; in the large intestine, colon-residing bacteria achieve the highest cell densities recorded for any ecosystem (O'Hara and Shanahan, 2006).

The microbial content of the GIT changes along its length, ranging from a narrow diversity and low numbers of microbes in the stomach to a wide diversity and high numbers in the large intestine (Power et al., 2014). The adhesion capacity, enzymes, and metabolic activity of the GIT also contribute largely to this variation of bacteria (Patel et al., 2016). Furthermore, the microbiota's composition at the mucosal surface differs from that within the lumen and faeces, and the ratio of anaerobes to aerobes is lower at the mucosal surfaces than in the lumen (Shanahan, 2002).

Microbial cells are present in minimal numbers in the stomach and proximal small intestine (duodenum and jejunum) (Figure 1.5). They can not colonize these regions because of the acidic pH (stomach) and relatively rapid flow of the digesta (duodenum and jejunum). The total bacterial count in gastric contents is usually below 10^3 CFU/ml, while in the small intestine, numbers range from

approximately 10^4 CFU/ml contents to about 10^6-10^7 CFU/ml at the terminal ileum region (Tannock, 1999). The main factors limiting growth in the small bowel are the rapid transit of contents and bile and pancreatic juice secretion.

The human large intestine is a complex, heavily populated, and diverse microbial ecosystem. Several hundred species of bacteria are usually present. The majority are strict anaerobes, with typical numbers of about 10^{11}-10^{12} CFU for every gram of colonic content (Salminen et al., 1998).

Resident bacteria, the microbiota, outnumber human somatic and germ cells 10-fold (O'Hara and Shanahan, 2006) and in its entirety is estimated to contain 150-fold more genes than the human genome (Power et al., 2014). The entire human intestine is colonised by 10^{13} to 10^{14} microorganisms, the vast majority of which belong to the phylum *Firmicutes* (including *Clostridium*, *Enterococcus*, *Lactobacillus* and *Ruminococcus*) and to the phylum *Bacteroidetes* (including *Bacteroides* and *Prevotella* genera), which constitute over 90% of the totality known intestinal microorganisms and 30% of the total anaerobic population. The others are mainly *Actinobacteria*, *Proteobacteria*, *Verrucomicrobia*, and *Fusobacteria* (Guarner et al., 2017). Although bacteria predominate, *archaea* and *eukarya* are also represented (O'Hara and Shanahan, 2006).

1.4.1 Functions of intestinal microbiota

"The flora has a collective metabolic activity equal to a virtual organ within an organ" (O'Hara and Shanahan, 2006).

Co-evolution of the host and flora implies mutually beneficial interactions, defined as a symbiotic relationship by Guarner et al. (2017); hence any variation of these balance, known as dysbiosis, may lead to detrimental responses and chronic inflammatory disease (Shanahan, 2002).

Symbiotic relationships can be described as (Hill and Artis, 2010):

- mutualistic: both species gain a benefit from the interaction;
- parasitic: one member benefits and the other is harmed;
- commensal: one member benefits, and the other is unaffected.

The microbiota offers many benefits to the host through a range of physiological, structural, and metabolic functions such as shaping the intestinal epithelium, harvesting energy, protecting against pathogens, and regulating host immunity (Thursby and Juge, 2017).

The main protective function of intestinal bacteria is to provide a fortification of barrier role that prevents pathogenic bacteria from invading and colonizing the GIT by competitive exclusion, such as the occupation of attachment sites, competition for receptors, consumption of nutrient sources, and production of antimicrobial substances (e.g., bacteriocins, lactic acid) (Sekirov et al., 2010).

The primary metabolic activity of the large-intestinal microflora is the breakdown, through fermentation, of carbohydrates, not digested in the upper gut, to SCFAs, which are then rapidly absorbed (Salminen et al., 1998). In this way, the host can salvage energy from indigestible food components that would otherwise be lost by excretion from the digestive tract.

The major substrates available for fermentation are starches that enter the colon (resistant starch), the non-starch polysaccharides (e.g., cellulose, hemicelluloses, pectins, and gums), and oligosaccharides (Cummings and Macfarlane, 1997). By producing SCFAs such as acetate, propionate, and butyrate, the resident bacteria positively influence intestinal epithelial cell differentiation and proliferation, and mediate other metabolic effects. In addition, gut bacteria are involved in vitamin synthesis (especially vitamins B_{12} and K) (Woodmansey, 2007) and modulation of fat deposition (O'Hara and Shanahan, 2006).

Regarding metabolic effects, the microbiota shapes the development of the immune system, and the immune system in turn shapes the composition of the microbiota (Nicholson et al., 2012). The sheer number and the large intestinal surface area of intestinal bacteria present a persistent threat: they pose a constant risk of invasion (Thursby and Juge, 2017). The opportunistic invasion of host tissue by resident bacteria can break down the symbiotic host-microorganism relationship and contribute to pathologies such as bacteraemia or chronic inflammation. To maintain homeostasis, the GIT limits the host immune system's exposure to the microbiota by recruitment of a multifactorial and dynamic intestinal barrier. The barrier comprises several integrated components including physical (the epithelial and mucus layers), biochemical (enzymes and antimicrobial proteins), and immunological (IgA and epithelia-associated immune cells) factors (Hooper and MacPherson, 2010).

The microbiota strongly influences the host immune system, and it contributes to the development and differentiation of the mammalian immune system. Different studies have been proposed using germ-free (GF) animals, mostly mice, to understand the connections between intestinal bacteria and stimulation of immune system. Different effects on the structural and functional development of the immune system have been revealed comparing GF mice and "microbiota-colonized animals". The most frequently detected are (Maslowski and MacKay, 2011; Maynard et al., 2012; Nicholson et al., 2012; Sekirov et al., 2010):

- GF animals contain abnormal numbers of several immune cell types and immune cell products;
- deficits in local and systemic lymphoid structures and tissues development, such as the spleen, thymus, and lymph node in GF mice;
- the above-mentioned structural abnormalities have been detected mostly near the mucosal interface, suggesting that interactions with specific microbes communities directly modulate

the development of these GALTs. Different types of GALT known as isolated lymphoid follicles, Peyer's patches of the distal ileum and mesenteric lymph nodes are minimally present and smaller in GF mice;

- GF mice showed an irregular intestinal epithelial cell morphology that includes longer villi and shorter crypts in comparison with that observed in mice with natural microbiota;
- the hyperactivity of innate immune response in GF mice, such as macrophages, presents higher concentrations of lysosomal enzymes.

These different studies demonstrate that the microbiota participates in the maturation of the immune system and suggests that specific events in association with microbial colonization may be important in the development of a normal immune system in a healthy individual (Gensollen et al., 2016).

Widespread antibiotic administration, while working on targeted infections, perturbs the intestinal microbiota. Deep 16S rDNA sequencing, performed after antibiotic treatment, has revealed dramatic and long-term changes to the intestinal microbiota that have implications for immune defense (Brandl et al., 2008; Ubeda and Pamer, 2012). Regarding this, Bohnhoff et al. (1954) revealed that streptomycin administration alters the gut microbiota and contributes to mice susceptibility to infection with *Salmonella* spp. Similarly, $\leq 10^1$ *Salmonella enteritidis* were sufficient to kill GF guinea pigs, whereas 10^9 bacteria were required to kill animals with complete intestinal microflora (Freter, 1955).

Furthermore, this fragile balance between microbiota and host can be affected by diet. Extreme "animal-based" or "plant-based" diets result in alterations of the gut microbiota in humans (Thursby and Juge, 2017). Foodborne microbes from both diets transiently colonized the gut, including bacteria, fungi, and even viruses (David et al., 2014). A "plant-based diet" is rich in grains, legumes, fruits, and vegetables, while an "animal-based diet" comprises meats, eggs, and cheeses. Comparing these two different diets, the latter one resulted in significantly lower levels of the products of carbohydrate fermentation, a higher concentration of the products of amino acid fermentation, and increased expression of genes associated with degradation of polycyclic aromatic hydrocarbons, which are carcinogenic compounds produced during the charring of meat (Turnbaugh et al., 2009).

Due to the fastidious nature of many probiotic bacteria, especially *S. thermophilus*, survival in sufficiently amount during passage through the above-mentioned environment of human GIT remains a major challenge for effective delivery of these beneficial bacteria (Annan et al., 2008).

1.4.2 *S. thermophilus* survival in the gastrointestinal tract conditions

The ability to survive the GIT transition is considered a key feature of probiotic strains to preserve their expected health-promoting effects (FAO/WHO, 2006) and to be assessed most studies proposed

the recovery of viable cells in fecal samples after oral administration. The concentration of probiotics needed to obtain a health effect is estimated as ≥ 10^6 CFU/ml in the small bowel and ≥ 10^8 CFU/ml in the colon (Minelli and Benini, 2008).

Martinović et al. (2020) analyzed different studies, and suggested that the recovery and identification of *S. thermophilus* is not always correctly performed. Primarily, other *Streptococcus* spp. that inhabit the human GIT could interfere, or could be misidentified, with the correct identification of *S. thermophilus* in stool samples. In particular, *S. salivarius* has been recovered from fecal samples of infants, thus revealing that this specie is one of the primary inhabitant of the intestinal microbiota (Favier et al., 2002; Kawamura et al., 1995). Moreover, molecular tools developed for the identification of *S. thermophilus* are based on species-specific polymerase chain reaction (PCR) primers targeting the 16S rDNA gene sequence. In a comparative genomic analysis performed by Delorme et al. (2015), the 16S rDNA gene sequence has been found not specific enough because of a high identity among those sequences in *S. thermophilus*, *S. salivarius* and *S. vestibuilaris* species (99.8% between *S. thermophilus* and *S. salivarius*; 99.6% between *S. thermophilus* and *S. vestibularis*) (Delorme et al., 2015).

Moreover, contradictory results from different studies showed by Martinović et al. (2020) on the ability of *S. thermophilus* strains to survive GIT conditions may be due to differences in the absolute number of live cells administered (Fenster et al., 2019).

Also, viability has been classically determined by plate counting; however it can shows disadvantages, such as an underestimation of viable bacteria due to the irregular distribution of microorganisms in the sample and the loss of culturability under stress conditions (García-Hernández et al., 2012a).

In conclusion, Martinović et al. (2020) recommended to create guidelines and protocols for the correct recovery and identification from stool samples of orally administered probiotics. In this sense, many *in vitro* and *in vivo* (human trial) studies have been performed with contradictory results, as shown below.

1.4.2.1 *In vitro* experiments

The Expert Consultation (2001) stated that "proper *in vitro* studies should establish the potential health benefits of probiotics prior to undertaking *in vivo* trials" (FAO/WHO, 2006). *In vitro* experiments contribute to a better understanding the influence of pH and bile salts concentrations on bacteria survival and physiology.

Numerous *in vitro* multi-compartmental models, which simulate different parts of the human gastrointestinal tract, have been developed to study the survival rate of potential probiotic strains. For

instance, Uriot et al. (2016) proved the survival of *S. thermophilus* using the dynamic TIM model (TNO gastrointestinal model), which closely simulates the physicochemical conditions found in the human stomach and small intestine, such as body temperature, changes in pH, transit time, sequential supply of digestive enzymes and bile salts as well as passive absorption of nutrients and water (Uriot et al., 2016).

Junjua et al. (2016) tested 30 *S. thermophilus* strains of different origins for their capacity to resist stresses representative of the GIT and to adhere to enterocytes. Resistance to the acidic environment was determined at pH 2 and 4. The first pH value corresponds to the stomach pH observed during inter-prandial phases, whereas the second is often used to determine the *in vitro* capacities of survival, and it is considered more realistic since bacteria are generally incorporated in the food-bolus which buffers the pH of the stomach and protects them against direct exposure to extreme pH conditions (Both et al., 2010). At pH 2 no growth was detected for all of the strains, whereas all of the strains resisted pH 4. This outcome is in accordance with the ability of the tested strains to remain alive in milk at the end of fermentation when the pH is about 4.2-4.3 (Galia et al., 2009).

Zhang et al. (2020) selected ten different strains of *S. thermophilus*, showing strong resistance to simulated gastric juice: the survival rates of all strains were at a high level (> 95%), and there was no significant difference when pH was 5.0. Since these strains have been isolated from the pickle, it was completely reasonable that they were well adapted to pickle acid conditions with a pH of about 3.5 (Zhang et al., 2020).

Regarding the resistance to bile salt, thus conditions of the small intestinal tract, a high strain-dependent was observed in Junjua et al. (2016) and Iyer et al. (2010a). Adhesion property of a probiotic to intestinal mucosa is also regarded as a prerequisite for probiotics. *In vitro* models are based on adhesion to tissue culture cell lines such as Caco-2 and HT-29, which resemble the enterocytes of human small intestine, and to human intestinal mucus (Fernández De Palencia et al., 2008). In the study performed by Junjua et al. (2016) only 4 out of the 30 *S. thermophilus* strains tested showed an adhesion percentage higher than 8%.

Kebouchi et al. (2016) have investigated the effect of different concentrations of bile salts on *S. thermophilus* LMD-9 strain and concluded that it was resistant to bile salts up to 3 mM, and it was able to adhere to different intestinal epithelial cells (IECs) tested *in vitro*.

1.4.2.2 Human trials

To investigate probiotics survival, in particular *S. thermophilus*, in human GIT, human trials are the golden standard. However, these studies are costly and heavy to perform. FAO/WHO (2006) suggested that performing randomized, double-blind, placebo-controlled human trials to establish the

efficacy of the probiotic product; further, they have to be repeated by more than one recognized center for confirmation of results.

For example, Mater et al. (2005) investigated the survival of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* by culture analysis of feces from healthy subjects over a 12-day period of fresh yogurt intake containing 7.8 x 10^8 CFU/ml viable rifampin- and streptomycin-resistant strains of *S. thermophilus*. During the period of intake, viable *S. thermophilus* cells were recovered, by standard plating, from 32 out of 39 fecal samples (82%) at levels between 4.0 x 10^2 and 3.5 x 10^6 CFU/g of feces, suggesting that they can survive transit in the gastrointestinal tract (Mater et al., 2005).

Afterward, Elli et al. (2006) again confirmed that *S. thermophilus* could be retrieved from human feces of 20 healthy volunteers after commercial yogurt consumption for 1 week, although *S. thermophilus* has been detected from only one volunteer on day 7. They concluded that "we cannot exclude the possibility that a prolonged ingestion period or a larger amount of ingested yogurt, could positively affect the rate of *S. thermophilus* recovery from fecal samples" (Elli et al., 2006).

Later on, García-Hernández et al. (2012b) detected viable cells of *S. thermophilus*, especially when the intake of yogurt was the highest of the study, in 30% of the total number of samples analyzed by applying a direct viable count-fluorescence in situ hybridization (DVC-FISH) method to fecal samples. During consumption, the concentration of viable *S. thermophilus* in the feces rapidly increased to reach a plateau. In contrast, in the post-administration period, one or two weeks after suspension of yogurt consumption, there was a significant decrease in the number of positive samples, and *S. thermophilus* counts dropped below the detection limit (García-Hernández et al., 2012b).

Similarly, Venturi et al. (1999) described the recovery of *S. thermophilus* after oral administration of a multi-strain probiotic preparation, containing one strain of *S. thermophilus*, by standard plating, concluding that it is capable of colonizing the gut and the duration of colonization was evident only during the drug's administration (Venturi et al., 1999).

Subsequently, Brigidi et al. (2003) detected *S. thermophilus* from human fecal samples after 3 days of intake of the pharmaceutical preparation VSL#3 (VSL pharmaceuticals Inc., Fort Lauderdale, USA) or yogurt, and it persisted for 6 days after the treatment suspension. Similarly, Mimura et al. (2004) demonstrated, through PCR analysis, that after administration of VSL#3, *S. thermophilus* was detected at higher levels in all patients treated.

The above-mentioned studies showed that *S. thermophilus* can survive the passage through the human GIT and can be detected in the feces of volunteers after consumption. However, the count of *S. thermophilus* decreased after administration, compared with other LAB that can be found longer in the feces (Venturi et al., 1999).

Contradictory results have been proposed by Del Campo et al. (2005). One hundred fourteen young volunteers took part in an experiment, with daily consumption of commercially produced fresh yogurt (375 g). Detection of yogurt LAB, especially *S. thermophilus*, in total fecal DNA by bacterial culture and PCR assay was consistently negative, suggesting that a substantial multiplication of yogurt bacteria in the small intestine is not expected to occur. The researchers concluded that "the presumed probiotic effect of yogurt should depends on the frequency of ingestion" (Del Campo et al., 2005).
It must be noted that none of these studies *in vivo* has investigated the survival of the bacterium when ingested alone (only within a yogurt or a preparation of different strains of probiotic bacteria), and survival to GIT conditions is strictly strain specific.
As reported above, according to the guidelines suggested by the FAO/WHO Expert Consultation, to be effective for use in foods, probiotic cultures must be capable of surviving passage through the digestive tract, possess the capability to proliferate in the gut, withstand processing conditions and survive in sufficient numbers (10^8 CFU/g) in the product during shelf-life storage (Minelli and Benini, 2008).
Several methods have been proposed to enhance the viability and to increase the resistance of sensitive probiotic microorganisms against adverse conditions, such as the selection of acid- and bile-resistant strains, stress adaptation system, incorporation of micronutrients (peptides and amino acids), and microencapsulation (Anal and Singh, 2007; Rokka and Rantamäki, 2010).

1.5 Microencapsulation technology

Microencapsulation (ME) is defined as the "technology of packaging solids, liquids or gaseous materials (active agent) in small capsules (wall material) that can release their contents at controlled rates under the influences of specific conditions over prolonged periods of time" (Anal and Singh, 2007). Also, encapsulation has been defined as a mechanical or physicochemical process to entrap active sensitive agents within a carrier material and as a useful tool to improve the delivery of bioactive molecules (e.g., antioxidants, minerals, vitamins, phytosterols, lutein, fatty acids, lycopene) and living cells (e.g., probiotics) into foods (Nedovic et al., 2011).
This technology is useful for different sectors:

- food industry: to encapsulate ingredients for functional food and nutraceuticals. The use of sweeteners encapsulated such as aspartame and flavours in chewing gum are well known (Gibbs et al., 1999);

- pharmaceutical sector: for drug and vaccine delivery. For example, Anal and Stevens (2005) develop multilayer beads, obtained through ionotropic gelation, for controlled delivery and release of the antibiotic ampicillin;
- agriculture, textiles, and paper industries (Timilsena et al., 2020).

In the food industry, the microencapsulation process can be applied for a variety of reasons, which have been summarized by Champagne and Kailasapathy (2008) and Rokka and Rantamäki (2010) as follows:

- stabilize the active content or food ingredients;
- provide barriers between sensitive bioactive materials and the environmental stressors. For instance, the capsules may allow the content pass through the adverse pH conditions of the stomach or can protect from light, cold shock (induced by process conditions such as deep freezing and freeze-drying), and heat shock (caused by process conditions such as spray drying);
- prevent reaction with other components in food products such as oxygen or water, and therefore control the oxidative reaction;
- provide a sustained or controlled release (both temporal and time-controlled release);
- mask bad flavours, colours, or smelling, such as unpleasant feelings during eating (e.g., bitter taste and astringency of polyphenols);
- extend the shelf-life and improve stability in final products and during processing;
- protect components against nutritional loss while increasing their bioavailability;
- immobilize cells or enzymes in food processing applications, such as fermentation and metabolite production processes, or improve the homogeneous distribution of cells throughout the product;
- modification of the physical characteristics to facilitate handling (e.g., converting the liquid active agent into a powder).

A microcapsule consists of a small vesicle or particulate with a semipermeable, spherical, thin, and strong membrane surrounding a solid/liquid core size (Fang and Bhandari, 2010). Beads without coating can also be considered as microcapsules in a broad sense.

As particle size affects texture, thus the sensory properties of food products, the capsules have to range from a diameter of few microns to several millimeters (Wandrey et al., 2010).

The encapsulated substance can be pure material or a mixture, and it is often called the core, fill, active, internal, or payload phase. On the other hand, the packaging material is called coating, wall, capsule, membrane, carrier, shell, external phase, or matrix (Fang and Bhandari, 2010; Jan Zuidam and Shimoni, 2010). For example, in nature, eggshells, plant seeds, bacterial spores, skin, and

seashells can be considered natural capsules, which allow protection of the contents from the environment while allowing small molecules to pass in and out of the membrane (Gibbs et al., 1999). Many morphologies can be distinguished with encapsulation technology, but two major types of encapsulates are more commonly identified (Schrooyen et al., 2001) as shown in Figure 1.6:

- mononuclear capsules or reservoir type: which have a single active core enveloped by a shell. This type is also called single-core, mono-core, or core-shell type;
- aggregates or matrix type: which have many cores, in the form of small droplets or more homogeneously distributed over the encapsulate, embedded in a matrix.

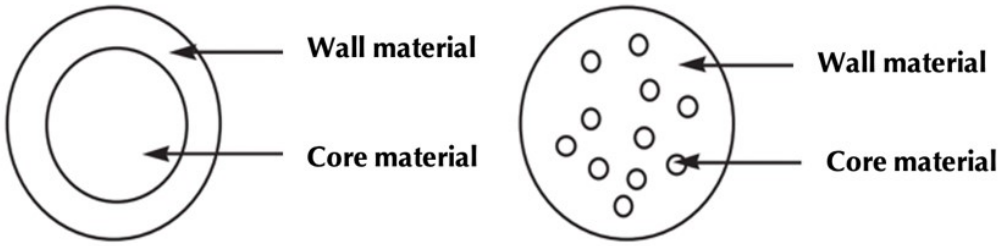


Figure 1.6 - Two major forms of encapsulation: mononuclear capsule or reservoir type (left) and aggregate or matrix type (right) (Fang and Bhandari, 2010).

1.5.1 Microencapsulation of probiotics

Nowadays, probiotics are the driving force in the design of functional foods, particularly in dairy products. Therefore, it is essential to improve cell survivability during GIT passage, and the formulation of these probiotics into microcapsules seems to be a promising emerging solution and an opportunity to control the release of these cells in their target, the gut (Cook et al., 2012). De Vos et al. (2010) claimed that microencapsulation of many probiotics may be mandatory for achieving the promised health benefits.

However, probiotics present two limiting factors when considering encapsulation: the size (typically between 1 and 5 μm of diameter), which immediately excludes nanotechnological approaches of encapsulation, and the cells must be kept alive (Champagne and Fustier, 2007). This latter point is crucial in selecting the appropriate probiotic encapsulation technology.

According to Anal and Singh (2007), the ME of probiotics and the addition into foods and beverages offers many technological benefits, beyond the improvement of viability of cells (Table 1.4).

The number of cells in beads or microparticles can be quantified, allowing the dosage to be readily controlled, and the narrow size and shape distribution can confer consistent performances (e.g., metabolite exchange) (De Prisco et al., 2017).

Moreover, cryo- and osmo-protective components can be incorporated into the matrix, enhancing cells' survival during processing and storage.

In addition, the coating layer can have desirable dissolution properties: microcapsules can be engineered to release the active core, which permits delayed release of the cells or release upon, for example, a change in pH (Anal and Stevens, 2005). There are many different ways of opening microcapsules, including heat shock fracture by the dissolution method, solvation, and applying suitable pressure (Riaz and Masud, 2013). Release properties are mainly based on particle size, shape, and material characteristics (Burey et al., 2008).

Sultana et al. (2000) reported that alginate concentration and capsule size can affect the survivability of probiotic: they are directly proportional to cell viability, whereas the initial cell numbers do not affect bacterial death rates. According to Chan and Zhang (2005), the cell release in alginate-based microencapsulation is primarily due to the erosion of the alginate gel layer. In particular, increasing the β-D-mannuronic acid (M) content of alginate, the gel structure formed becomes softer, more elastic, but less porous, and it can be dissolved more easily (Tønnesen and Karlsen, 2002).

A coating material may also be designed to open in the specific areas of the body: since the intestine provides the right conditions for probiotics to survive and multiply, release of cells into this site leads to probiotics settlement and activity. Mortazavian et al. (2007) found that when the capsules are opened in the small intestine, the released cells are expected to arrive in the large intestine.

Regarding the synbiotic interactions between probiotic bacteria and prebiotic, Etchepare et al. (2015) reported that the combination of alginate with prebiotics offers enhanced protection for probiotics in food systems. This can be explained by the fact that prebiotics form three-dimensional microcrystals networks that interact together, forming small aggregates that contribute to better protection for probiotics. The prebiotics applied in co-encapsulation (probiotic and prebiotic together) technology are fructooligosaccharides, inulin, and resistant starches (Etchepare et al., 2015). Their application is useful especially when invasive techniques that can decrease cell viability, such as spray or freeze-drying are used for the microencapsulation (De Prisco and Mauriello, 2016).

According to Sultana et al. (2000), co-encapsulation of probiotic bacteria with prebiotic improved entrapping of viable bacteria as compared to bacteria encapsulated without the prebiotic molecule. In particular, Hi-Maize starch (resistant starch) was used at different concentrations in the encapsulation procedure to provide prebiotics to the encapsulated probiotic bacteria. In conclusion, starch and alginate have a synergic interaction in gelation and prebiotic may help in providing additional protection to the entrapped bacterial cells (Sultana et al., 2000).

In general, for encapsulation, probiotic bacteria are grown in their optimal culture conditions, after which they are centrifuged and used in suspension form or as freeze-dried powder (Rokka and

Rantamäki, 2010). The ideal microencapsulated probiotic as a commercial product would either be a dry powder, with ease of storage and long shelf-life, or a wet gel with long-term stability in a food product (Cook et al., 2012).

Materials used for encapsulation of probiotic must be food-grade, biodegradable, and able to form a barrier between the internal phase and its surroundings (Nedovic et al., 2011). The most commonly used constituents in encapsulation of probiotic bacteria include polysaccharides originating from seaweed (κ-carrageenan, alginate), other plants (starch and its derivatives, gum arabic), bacteria origin (gellan, xanthan), and animal proteins (caseins and whey proteins, gelatin) (Rokka and Rantamäki, 2010).

Table 1.4 - Beneficial effects derived from the microencapsulation of lactic acid cultures in alginate matrices. Benefits are gained both for the production of probiotics and their use in nutraceutical and food applications (Champagne and Fustier, 2007; Mortazavian et al., 2007).

Benefit	Product
Facilitates the production of oxygen-sensitive cultures	Dried probiotic culture
Facilitates the recovery of centrifugation-sensitive cultures	Dried probiotic culture
Facilitates the recovery of high EPS-producing cultures	Dried probiotic culture
Less contamination problems	Dried probiotic culture
Cultures can be air-dried	Dried probiotic culture
Improved survival on exposure to gastric solutions	Nutraceutical
Improved survival on exposure to bile solutions	Nutraceutical
Improved stability during storage in dried form	Nutraceutical
Improved acidification rate	Dried sausages
Improved survival on heating	Biscuits, powder
Improved survival on freezing	Ice cream, milk-based medium, cranberry juice
Improved retention in the finished product	Cheese
Protection against bacteriophages	Fermented milks
Protection against yeast contaminants	Fermented milks
Improved survival during storage	Yoghurt, mayonnaise, milk

The techniques most commonly used in the microencapsulation of probiotics are emulsion, extrusion, and spray drying. Other less common methods are adhesion to starch and matrix encapsulation.

1.6 Microencapsulation methods

There are several techniques available for encapsulation of food compounds. They differ depending on the nature of the core and final use of capsules obtained.

First of all, to select an encapsulation system, it is needed a physicochemical understanding of the encapsulation mechanisms: possible interaction of the active core with the external phase, stability of the payload phase, and release actions from the matrix (Timilsena et al., 2020).
In general, three steps are required:

1. formation of the wall around the core material: primarily making droplets of the active (in gas, liquid, or powder form) and subsequently surrounded by carrier material in a gas or liquid phase via different physic-chemical processes;
2. ensuring that leakages, fissures do not occur;
3. ensuring that undesired materials are kept out.

Encapsulation techniques applied to the food industry include spray-drying, fluid bed coating, spray-chilling or spray cooling, injection, extrusion, emulsification, coacervation, co-extrusion, inclusion complexation, liposome entrapment, centrifugal extrusion, encapsulation by a rapid expansion of supercritical fluid, freeze- or vacuum drying, and nanotechnological approaches (Timilsena et al., 2020).

1.6.1 Emulsion technique

Emulsification is defined as a process of dispersing one liquid, in which the active component is included, in a second immiscible one. It is also known as internal ionic gelation or a two-phase system. In most cases, the encapsulating agent is a molecule already present in the food (Augustin and Hemar, 2009).
In this method, a small volume of cell/polymer slurry (dispersed or discontinuous phase) is added to the large volume of vegetable oil (continuous phase) such as sunflower, soy, corn, or light paraffin oil (Iravani et al., 2015). Subsequently, calcium chloride is added to the emulsion of water droplets of alginate (slurry) solution and active in vegetable oil. This results in the "break-up" of the emulsion, and microbeads are formed by the gelation of the alginate droplets (Jan Zuidam and Shimoni, 2010). This technique has been successfully applied for the microencapsulation of LAB.
As an example, Sheu and Marshall (1993) developed a method to entrap bacteria using a two-phase (water/oil) system. The encapsulation material, 3% of sodium alginate, has been first mixed with the microbial cells and suspended in an oil bath containing 0.2% Tween 80 as the emulsifying agent. The water/oil emulsion has been broken by adding $CaCl_2$, forming calcium alginate beads containing the cells (Sheu and Marshall, 1993).
The emulsion technique has the benefit of producing tiny (25 μm-2 mm) microcapsules, representing an advantage in laboratory-scale production, as particular equipment is not necessary to produce these tiny particles (Mokarram et al., 2009).

However, this technique is more expensive than the extrusion method due to the need to use vegetable oil for emulsion formation (Mortazavian et al., 2007).

1.6.2 Extrusion technique

The extrusion technique is also called droplet or external ionic gelation.

The method consists of the following stages (Nedovic et al., 2011):

- preparation of hydrocolloid solution/aqueous solution of polymer (most often this is 0.6-3 w/v % sodium alginate) and active/core (e.g., probiotic cells);
- extrusion of the cell suspension through syringe needle or nozzle in a way that the resulting droplets directly drip into the hardening solution/gelling bath (in case of alginate, a gelling bath is 0.05-1.5 M $CaCl_2$ solution).

Generally, the $CaCl_2$ coagulation solution consists of multivalent cations (e.g., calcium in the form of calcium chloride).

The dripping tool can be simply a pipette, a syringe, a vibrating nozzle, a spraying nozzle, jet cutter, or atomizing disk. However, the choice of the utensil is crucial since the nozzle or needle size used to drip the solution affects the size of the microcapsules, and the survival of cells in beads is higher with larger beads. Also, the diameter of the obtained beads and hence the cells' survival rate is found to increase as the concentration of sodium alginate increases (Lee and Heo, 2000).

Moreover, the size of the droplets depends upon the flow rate of the solution, and the viscosity of the solution, besides the distance between the syringe and the solution of calcium chloride (Burey et al., 2008). Generally, the diameter of capsules obtained with this method ranges from 500 μm to 3 mm, and therefore the microcapsules are larger than those formed in the emulsion method (Mortazavian et al., 2007).

In general, it is a simple and inexpensive method with gentle operations, which makes cell injuries minimal and results in more uniformly shaped microcapsules than achieved by the emulsion technique.

Extrusion technologies have many advantages for the encapsulation of microbes. It is relatively soft, and the procedure does not involve deleterious solvents. Also, the extrusion technique promotes a relatively high viability of probiotic cells (Iravani et al., 2015; Mortazavian et al., 2007; Rokka and Rantamäki, 2010). Extrusion technologies are also applied for flavors, enzymes, and proteins (De Vos et al., 2010).

One possible disadvantage is the difficulty to be scaled up. This procedure may not be applied for large-scale production, and it may be suitable only for laboratory-scale processes. Despite this, multi-nozzle arrays have been developed by industry to scale up extrusion operations (Cook et al., 2012).

Encapsulating materials used in extrusion technology include gelatin, sodium alginate, carrageenan, starches, cellulose derivatives, gum acacia, fats and fatty acids, waxes, and polyethylene glycol (Wandrey et al., 2010).

1.6.2.1 Alginate

The most common encapsulation agent is alginate due to its particularly mild gelling conditions, GRAS status, and lack of toxicity. Alginates or algin is a generic term for the salts and derivatives of alginic acid which are derived primarily from marine brown algae or bacterial sources (Cook et al., 2012). They are commercially available as Mg^{2+},Ca^{2+},Sr^{2+},Ba^{2+}, and Na^{+} salts (Gombotz and Wee, 2012). Sodium alginate is an example of water-soluble alginate (Wandrey et al., 2010).

Alginates are copolymer, structurally formed by linear unbranched natural polysaccharides made up of β-D-mannuronic (M) and α-L-guluronic acid (G) residues joined linearly by (1-4)-glycosidic linkages (Annan et al., 2008). Notably, due to the presence of carboxylic acid groups on both monomers, alginate carries a negative charge above its pKa (3.3-3.5) (Cook et al., 2012).

The intramolecular electrostatic repulsion between the contiguous negative charges of each monomer forces alginate molecules into an extended random coil conformation. Even at low concentration, alginate solutions present high viscosity due to this coil conformation (Wandrey et al., 2010).

The ability of alginate to form gels is due to the interactions between divalent cations (e.g., calcium, cadmium, or zinc) and four guluronic acid (G) residues, forming what has been described as an "egg-box structure" (Etchepare et al., 2015). In other words, the polymer chain adopts a characteristic zigzag shape, as shown in Figure 1.7. This mechanism is known as ionotropic gelation.

The gelation occurs when it is produced a zone of union (interchain association) between the carboxyl groups of two pairs of two consecutive acid α-L-guluronic (G) units, each pair belonging to different alginate chains, through the coordination of a Ca^{2+} cation forming a stable junction zone (Etchepare et al., 2015; Sikorski et al., 2007). This linkage process is known as cross-linking (Gombotz and Wee, 2012). The importance of the G units in this process is highlighted by the fact that the gel strength is directly related to the total content of G units (Sikorski et al., 2007).

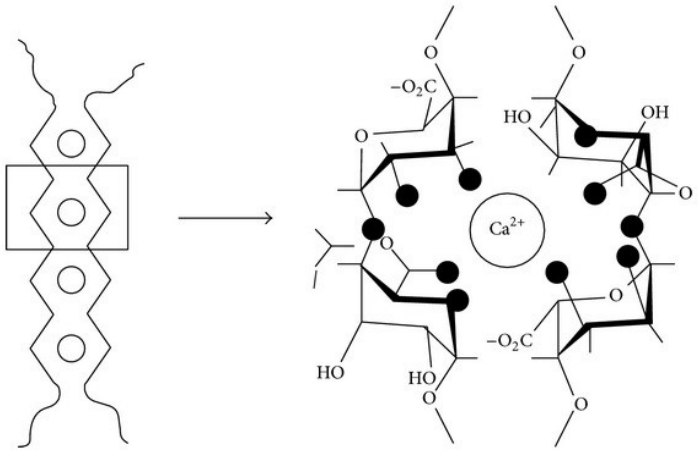


Figure 1.7 - Schematic drawing and calcium coordination of the egg-box model: two consecutive guluronate chains in calcium alginate junction zones. Dark circles represent the oxygen atoms involved in the coordination of the calcium ion (Sikorski et al., 2007).

1.6.2.2 Gelatin

Gelatin differs from the hydrocolloid (alginate) discussed above in that it is derived from an animal protein, collagen, via controlled acid or alkaline hydrolysis (Burey et al., 2008). Common gelatins are mainly extracted from bovine (cow) and porcine (pig) sources, but other origins such as piscine (fish) and poultry may be used. These gelatins vary widely in their size and charge distribution (Armisen and Galatas, 2009).

Gelatin consists of 14% moisture, 84% protein, and 2% ash (Burey et al., 2008). The protein fraction consists of a heterogeneous mixture of single- or multi-stranded polypeptides, in which amino acids such as glycine, proline, and hydroxyproline are present in the most abundance (Wandrey et al., 2010). The general amino acid sequence is glycine-X-Y triplets, where X and Y are frequently proline and hydroxyproline amino acids (Figure 1.8). This repeating structure occurs over the entire polymer chain forming the characteristic triple helical structure in gelatin (Burey et al., 2008). In gel formation, triple helical segments form the basis for cross-linking and three-dimensional network formation (Figure 1.9): the coils undergo a coil-to helix transition, leading to thermoreversible gelation (Wandrey et al., 2010). It does not form beads but could still be considered as material for microencapsulation (Gbassi and Vandamme, 2012). Gelatin is amphoteric in nature, due to the presence of carboxylic and aminoguanidine groups (Saravanan and Rao, 2010), and for this, it shows the ability to have synergistic effects with anionic gel-forming polysaccharides such as gellan gum (Krasaekoopt et al., 2003) or alginate (Annan et al., 2008). The two mentioned polymers are miscible at pH > 6 because they both carry net negative charges. However, the net charge of gelatin becomes

positive when the pH is adjusted below its isoelectric point and causes a strong interaction with the negatively charged gellan gum and alginate (Huq et al., 2013; Mortazavian et al., 2007).

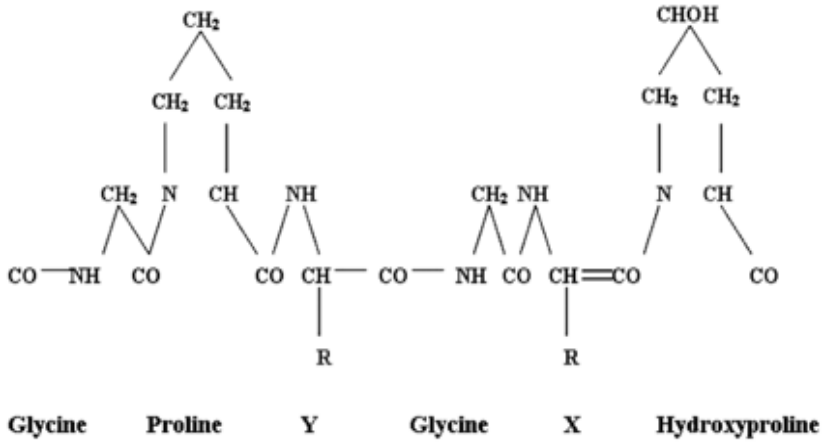


Figure 1.8 - Gelatin repeating structure responsible for triple helix structure (Duconseille et al., 2015).

According to Annan et al. (2008), encapsulation in alginate-coated gelatin microspheres significantly ($P < 0.05$) improved the survival of probiotic bacteria (*Bifidobacterium adolescentis*) in the harsh acidic conditions of simulated gastric juice in comparison to free cells.

Gelatin is easily solubilized in most polar solvents due to the presence of charged amino acids, forming colloidal solutions. This means that gelatin by definition is a hydrocolloid (Armisen and Galatas, 2009).

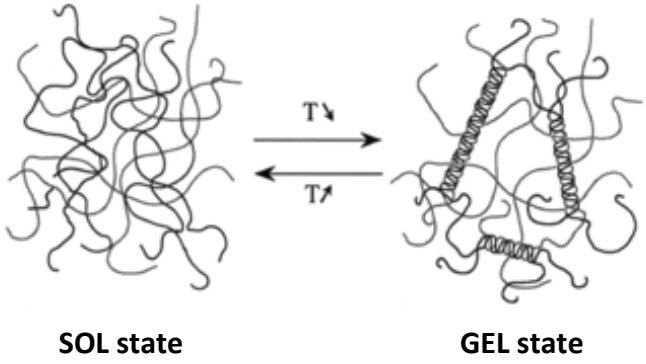


Figure 1.9 - Mechanism of gelatin gelation. A gelatin solution forms a gel upon cooling due to the formation of triple helices stabilized by intermolecular hydrogen bonds, while gel heating leads to the reverse process (Eysturskarð, 2010).

Due to the above-mentioned properties, the food industry is one of the major consumers of gelatins. For example, jellies, represent food products that take advantage of the thermoreversible gel formation and the "melt-in-the-mouth" texture of gelatin. In addition, other important nutritional and health properties are: high-quality source of protein, free of cholesterol and sugar, and safe (GRAS status) (Armisen and Galatas, 2009). Moreover, gelatin is easily digested and utilized by the human organism.

However, recent research has focused on finding a suitable alternative to gelatin microencapsulation because it is from animal sources, and this is not accepted by a specific group of consumers based on dietary preferences and religious beliefs.

1.6.3 Spray drying

Spray-drying is one of the oldest processes and the most widely used encapsulation technique in the food industry (Nedovic et al., 2011).

The principle of spray drying is dissolving, emulsifying, or dispersing the active core in an aqueous solution of the chosen carrier material. The mixture is subsequently atomized into a hot chamber (Jan Zuidam and Shimoni, 2010). This promotes rapid evaporation of the solvent (water). The capsules are obtained as a dry powder after separation from the drying air at the outlet at a lower temperature (Rokka and Rantamäki, 2010; Timilsena et al., 2020).

The spray drying technique is flexible, continuous, fast, and relatively economical. It produces particles of good quality, which size is less than 40 μm, very stable, thus allowing a significant increase in shelf-life, and when adequately performed, is highly reproducible (De Vos et al., 2010; Iravani et al., 2015).

However, this technology also has some disadvantages: the active component may be exposed and thus representing problems when considering the technique for encapsulation of probiotics, where the bacteria may leak into the product when some hydration occurs (De Vos et al., 2010). Another limitation is the high temperature required to facilitate water evaporation that is not compatible with the survival of all types of probiotics, reducing their viability in the final product (Rokka and Rantamäki, 2010).

Besides, once the capsules are obtained from other encapsulation techniques, this procedure can isolate them as a dry powder for ease of handling and storage. For this purpose, different drying procedures have been used, including spray drying, air drying, freeze-drying, and fluid bed drying (Cook et al., 2012).

In spray chilling or spray cooling, the active material to be encapsulated is mixed with the carrier and atomized by chilled or cooled air instead of heated air as in spray drying (Gibbs et al., 1999).

1.7 Aim of the thesis

Due to the fastidious nature of many probiotic bacteria, especially *S. thermophilus*, survival in sufficient amounts during passage through the human gastrointestinal tract (GIT) is a key factor to confer health benefit to the host. Among the different strategies proposed to protect bacteria cells, microencapsulation technique offers a great potentiality in the delivery of viable cells.

The aim of this project was to investigate the most suitable encapsulating agent for microencapsulation of the potential probiotic strain *S. thermophilus* TH982, isolated from a dairy environment. Using four types of substances to encapsulate bacteria cells, different parameters have been analyzed in order to evaluate the survival of viable cells to the encapsulation procedure, storage in skimmed milk, passage through simulated gastrointestinal tract and final released in the lower intestinal tract. The materials employed in the extrusion process for the formation of alginate-based capsules are represented by three categories: a polysaccharide (inulin), a protein (gelatin) and a human milk oligosaccharide (2'-fucosyllactose).

2 MATERIALS AND METHODS

2.1 Bacterial strain

A potential probiotic bacterium strain, *S. thermophilus* TH982, which has the characteristics of good potential EPS produces, great resistance to simulated gastrointestinal conditions, high extracellular folate production (Tarrah et al., 2018), was used as the target strain in this work. This strain was studied for hemolytic activity from the safety aspect, and it was detected as γ-hemolytic (i.e., without hemolytic activity).

According to Treu et al. (2014), TH982 has been isolated in the Campania region from whey and curd, respectively, obtained from mozzarella di bufala campana DOP cheese (pasta filata cheese) production. The strain was also characterized for genome sequence by Treu et al. (2014).

2.2 Bacteria and growth conditions

Pure culture of probiotic bacteria (*S. thermophilus* TH982) was obtained in freeze-dried form from the Department of Agronomy, Food, Natural resources, Animals and Environment, University of Padova, Italy.

Stock culture of *S. thermophilus* TH982 was stored at -80 °C in M17 broth (Sigma-Aldrich) containing glycerol (25% v/v) and was activated by inoculating the culture into 45 ml of M17 (Sigma-Aldrich) broth medium (42 g of M17 in 1 liter of distilled water) for 24 h at 37 °C. The reactivated cells were centrifuged at 5500 rpm for 5 min at room temperature, and subsequently, the supernatant was discarded to obtain bacteria pellets.

The cells were then washed twice by sterile distilled water and used in the encapsulation process. Fresh cell suspensions were prepared for each experiment and numerated by micro drop technique plating on M17 agar. Plates were incubated for 48 h at 37 °C.

2.3 Microencapsulation procedure

The agents used for the microencapsulation of *S. thermophilus* TH982 are represented by sodium alginate (S), a linear heteropolysaccharide of D-mannuronic and L-guluronic acid extracted from various species of algae, used as main encapsulating material (control) (Cook et al., 2012); gelatin (G) from porcine skin, a mixture of heterogenous polypeptides which has the ability to form thermoreversible gels (Burey et al., 2008); inulin (I), a linear polydisperse mixture of β-2-1 linked D-fructose molecules and a prebiotic, which resist hydrolysis by human small intestinal digestive

enzymes (Roberfroid, 2005); 2'-fucosyllactose (F), a human milk oligosaccharide, and a bioactive molecule which protect the breastfed infant from pathogen acting as a prebiotic (Kunz et al., 2000; Newburg et al., 2005).

The extrusion technique was carried out in a modified version described earlier by Mirzaei et al. (2012) and Liao et al. (2019).

All glassware and solutions used in the protocols were sterilized at 121 °C for 15 min.

The microencapsulating matrix of probiotic cells was prepared through the combination of 2% (w/v) sodium alginate with 2% (w/v) inulin, 2% (w/v) gelatin, 2% (w/v) 2'-FL, and sodium alginate alone used as control. For each capsule type, the different matrices were mixed, adjusted to the final concentration of 2%, and added to the TH982 pellet suspension.

The mixture was injected through a sterile syringe with a needle size of 450 µm of diameter into sterilized 0.3 M $CaCl_2$ hardener solution. The droplets formed gel spheres immediately. The distance between the bottom of the nozzle and the surface of the $CaCl_2$ solution was 10 cm (Figure 2.1).

After 30 min in the hardening solution ($CaCl_2$), the obtained capsules were collected by filtration and stored in 0.1% (w/v) sterile peptone water solution at 4 °C until further use.

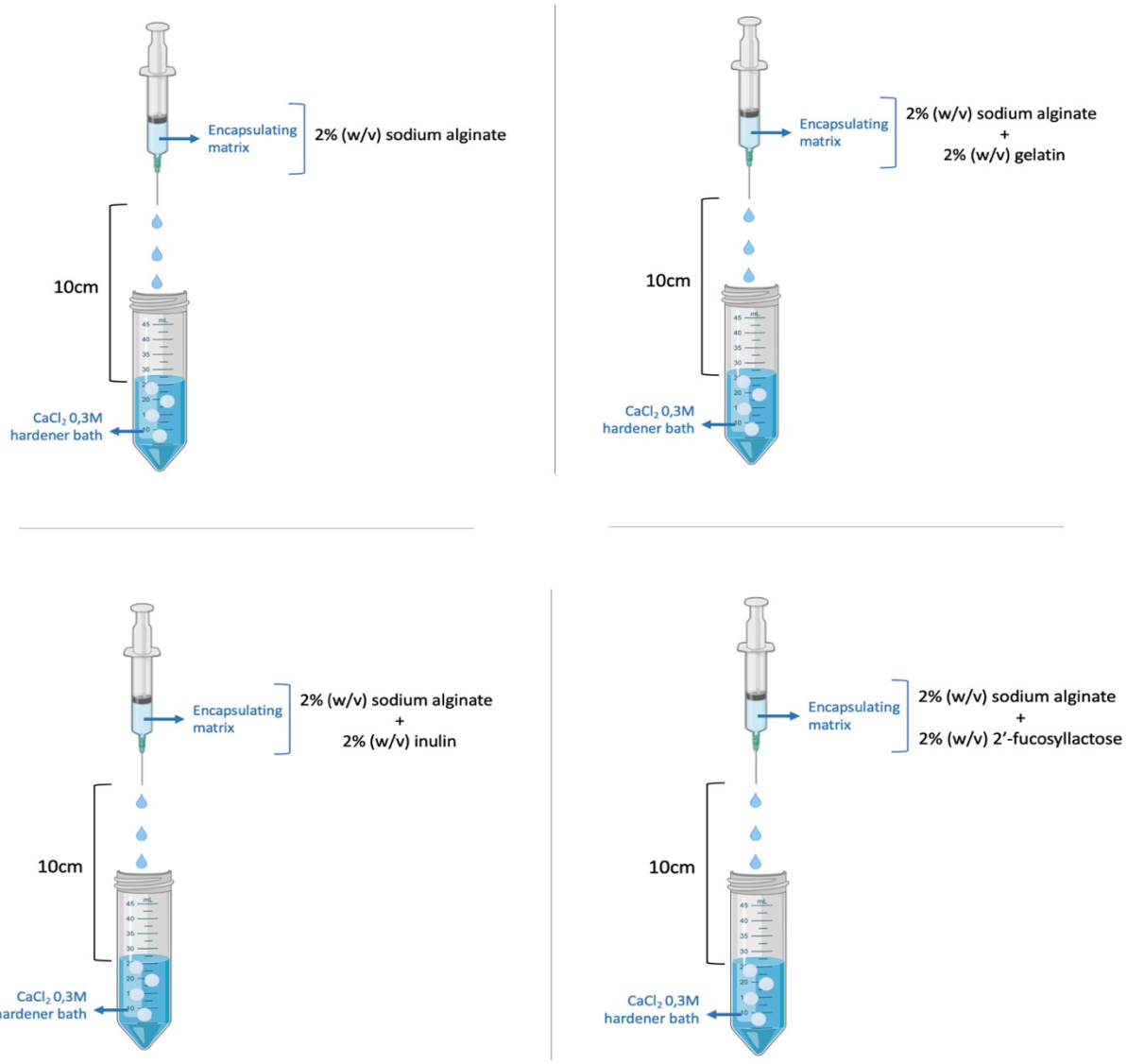


Figure 2.1 - Microencapsulation of bacteria cells with different concentration of encapsulating matrices using extrusion technique.

2.4 Morphology of capsules

The morphology of the microcapsules was examined using an optical stereo microscope (Olympus, Tokyo, Japan).

2.5 Encapsulation efficiency

To break the encapsulated polymers, 1 g of capsules was suspended in 9 ml of 10% (w/v) sterilized sodium citrate and homogenized for 1-2 min. To determine the viable concentration in capsule

solutions, before encapsulation, 1 ml of each type of capsule matrix was added to 9 ml of distilled water.

The homogenized samples were serially diluted with sterile distilled water, and the viable cells were determined and expressed as log colony-forming units per gram (log CFU/g) for capsules, and log colony-forming units per milliliter (log CFU/ml) for matrices, by plating 20 µl of the appropriate diluted bacterial cultures, with micro drop technique, on M17 agar plates and incubation at 37 °C for 48 h.

The encapsulation yield, or encapsulation efficiency (EE), was determined using the given equation (Chávarri et al., 2010):

$$\mathbf{EE\ \%= (N_1/N_0)\ x\ 100}$$

Where N_1 is the number of viable entrapped TH982 cells (log CFU/g) released after the encapsulation procedure, and N_0 is the number of viable cells (log CFU/ml) added into the mixture before encapsulation.

To perform the micro drop technique, 100 µl of each solution was added to 900 µl of sterile water, and serial decimal dilutions of the solution were performed down to 10^{-9}. Then, 20 µl aliquots of each dilution were spotted on the surface of M17 agar plates. The experiment was executed using three technical replicates.

2.6 Storage in skimmed milk

To determine the survival of encapsulated TH982 during the storage, 1 g of microcapsules was suspended in 9 ml of 10% (w/v) reconstituted skimmed milk as it is used for the preparation of most of the fermented dairy products (Singh et al., 2019), and stored under refrigerated conditions (4 °C) for different time intervals (0, 7d, 14d, 21d).

The survival of free (non-encapsulated) bacteria cells was determined by adding 9 ml of 10% (w/v) reconstituted skimmed milk to 1 ml of free cells as well.

At each time interval, 1 g of capsules was collected, centrifuged at 5500 rpm for 5 min, and the supernatant was discarded. The cells were then released in 10% (w/v) sodium citrate, serially diluted, and plated on M17 agar.

The survival was determined as the number of cells (log CFU/g) recovered during different storage time intervals considering the number of initial entrapped cells. The experiment was done using three technical replicates.

2.7 Survival under simulated gastrointestinal conditions

The survival of free cells and encapsulated cells was evaluated under *in vitro* simulated gastrointestinal conditions.

The simulated gastrointestinal conditions were obtained using a basic juice, gastric juice, and intestinal juice. The basic juice was prepared by dissolving (per liter): 1.12 g potassium chloride, 2.0 g sodium chloride, 0.11 g calcium chloride, and 0.4 g potassium dihydrogen phosphate in distilled water. The basic juice was sterilized by autoclaving at 121 °C for 15 min.

The simulated gastric juice (SGJ) and simulated intestinal juice (SIJ) were prepared using a modified method from Singh et al. (2019).

The gastric juice consisted of 0.01 g/l of swine pepsin (Sigma-Aldrich) and 0.01 g/l of swine mucin (Sigma-Aldrich) added directly to the sterile basic juice. The pH was adjusted to 2.5 with 1 N HCl, and the liquid was filter-sterilized using a 0.22 µm membrane filter (Sigma-Aldrich).

The intestinal juice contained (per liter of basic juice): 0.01 g pancreatin (Sigma-Aldrich), 0.08 g Ox-bile extract (Sigma-Aldrich) and 0.01 g lysozyme (Sigma-Aldrich). The pH was adjusted to 7.5 with 1 N NaOH, and the juice was filter-sterilized. Both gastric and intestinal juices were prepared fresh on the day of the experiment.

Overnight bacteria cells were obtained after two-times subculturing in M17 broth, and the capsules were prepared fresh as described above. One g of capsules was added to 9 ml of SGJ and incubated at 37 °C for 1 h to evaluate the survivability of TH982 using different encapsulation matrices. The experiment was performed for all different capsule types and the bacteria alone.

After the incubation period, the cells' viability was evaluated using the technique mentioned earlier in the storage section.

For the survival of TH982 under gastrointestinal conditions, 9 ml of SIJ was added following gastric juice incubation. Capsules and free cells with simulated gastric and intestinal juice were left at 37 °C for a further 2 h. After the incubation period, the cells' viability was evaluated using the technique mentioned earlier in the storage section. The experiment was performed using three technical replicates.

2.8 Release kinetics

The release kinetic study was carried out in intestinal fluid without enzymes.

The release of encapsulated *S. thermophilus* TH982 was evaluated according to Yongsheng et al. (2008), Wang et al. (2016) and Shi et al. (2013a) with some modifications.

A simulated intestinal fluid was prepared according to Shi et al. (2013a) (6.8 g/l of K_2HPO_4, 50 mM) and stirred until complete dissolution by using a magnetic stirrer. The pH was adjusted to 7.5 with 1 N HCl and subsequently sterilized by autoclaving at 121 °C for 15 min.

Microcapsules (1 g) from different matrices were added to 9 ml of SIJ pH 7.5 (without enzymes), and incubated at 37 °C using a 100 rpm rotator. At predetermined time intervals (initial, after 30, 60, 90, 120, 150, 180 min), 100 µl of solutions were taken from each capsule type, serially diluted, and plated using the micro drop technique on M17 agar to detect the number of released cells. The number of released cells was determined after 48 h of incubation at 37 °C and expressed as log CFU/ml of K_2HPO_4 solution.

2.9 Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA). Tukey's test was used as *post hoc* analysis by the GraphPad Prism software (version 7, GraphPad Software, Inc., San Diego, CA, United States). In general, results were considered significantly different when *P values* were lower than 0.05.

3 RESULTS AND DISCUSSION

3.1 Encapsulation efficiency

The encapsulation efficiency (EE) of the four microcapsule types is shown in Table 3.1.

The EE is a combined parameter that describes the survival of viable cells and the efficacy of entrapment during the encapsulation procedure (Heidebach et al., 2012). The efficiency of entrapping cells in capsules was different among two groups of encapsulating matrices, as reported in in Table 3.1.

The yield of TH982 cells co-encapsulated with the oligosaccharide 2'-FL (96.13%) was significantly higher ($P < 0.01$) than that of cells encapsulated with sodium alginate alone (91.07%), used as control. Furthermore, the yield of encapsulation with sodium alginate and inulin (98.61%) was similar (not statistically different) to that of 2'-FL.

The high EE derived from the combination of sodium alginate with oligosaccharide (2'-FL) and polysaccharide (inulin) might be due to the decrease in the porosity of the gel-beads and thus reduction in the leakage of entrapped TH982 cells (Liao et al., 2019). Besides, incorporating prebiotics as materials for encapsulation may better protect probiotics in food systems and the gastrointestinal tract (Sathyabama et al., 2014).

By contrast, the number of viable cells encapsulated in sodium alginate alone and in combination with gelatin indicated no significant difference, with EE 91.07% and 90.50%, respectively.

Table 3.1 - Encapsulation efficiency of *S. thermophilus* TH982 using different encapsulating matrix.

	ENCAPSULATION SOLUTION (log CFU/ml)	AFTER ENCAPSULATION (log CFU/g)	ENCAPSULATION EFFICIENCY (EE%)
S	9.46±0.04	8.61±0.19	91.07[a]
S+I	9.17±0.21	9.04±0.12	98.61[b]
S+G	9.39±0.09	8.50±0.04	90.50[a]
S+F	9.34±0.03	8.97±0.07	96.13[b]

Superscripts letters indicate statistically significant ($P < 0.05$) differences between different type of encapsulating matrix.

Values are the mean ± standard deviation (SD) of triplicate experiments (n=3).

S: sodium alginate; S+I: sodium alginate + inulin; S+G: sodium alginate + gelatin; S+F: sodium alginate + 2'-FL.

The main reason for an EE below 100% is linked to cell damage due to detrimental conditions caused by the encapsulation process itself, such as shear stress and use of concentrated solutes. Furthermore,

during time (30 min) required for hardening of capsules, a physical loss of cells can occur in significant numbers (Heidebach et al., 2012).

It should also be noted that a dissolution process is required to determine the number of viable cells in the microcapsules, and hence an incomplete disintegration can underestimate the EE value found. In particular, for the solubilization of alginate-based capsules, sodium citrate (10% w/v) was used, which is a chelating agent (such as phosphate and lactate) (Liao et al., 2019). It can chelate calcium from the egg box structure, leading to destabilization of alginate coating and effective release of cells (Annan et al., 2008).

3.2 Morphology of capsules

During the extrusion of the sodium alginate-based matrix into the $CaCl_2$ bath, wet spherical capsules were immediately formed by establishing cross-linkage between carboxyl groups and Ca^{2+} ions (Vaziri et al., 2018).

The images of the microcapsules entrapping the *S. thermophilus* TH982 indicate that all capsules were globular and irregular in shape with a rough surface and display a drop-like shape with a small tail (Figure 3.1). This result is probably due to the high surface tension of the hardening solution used ($CaCl_2$) that results in the imperfect sphere formation (El-shafei et al., 2018). However, no surface cavities or fractures were detected.

The capsules with sodium alginate and gelatin (B in Figure 3.1) showed a more dense structure, as confirmation of gelatin solution viscosity, due to electrostatic interactions between the amino groups of gelatin and carboxyl groups of alginate which enabled cells to resist to enzymatic and acidic hydrolysis (Vaziri et al., 2018).

The size range obtained might not be suitable for specific dairy product applications, and in the future, the evaluation of sensory characteristics of these capsules would be of interest.

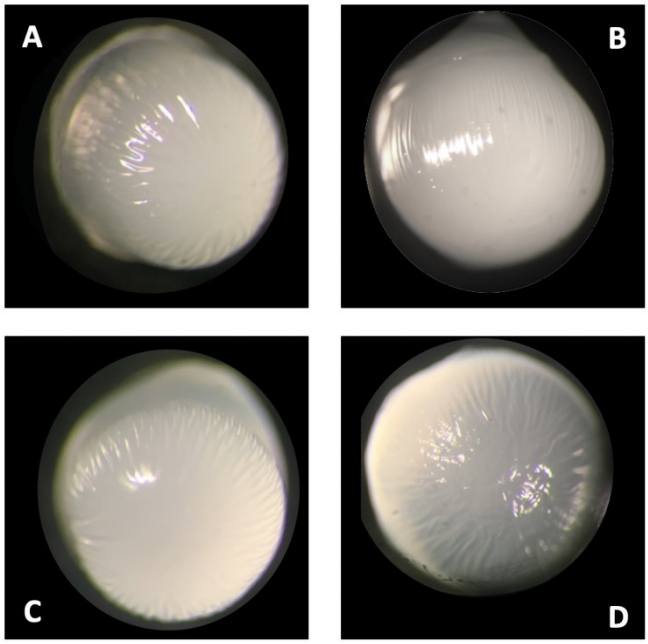


Figure 3.1 - Pictures taken by amplification of the samples with a stereo microscope; magnification 100X.

A: sodium alginate (S); B: sodium alginate + gelatin (S+G); C: sodium alginate + inulin (S+I); D: sodium alginate + 2'-FL (S+F).

3.3 Storage in skimmed milk

Results in Figure 3.2 illustrate that the viability of all the encapsulated *S. thermophilus* cells during storage in skimmed milk at 4 °C for 21 days showed little viable cell reduction from the initial count, which was around 10^9 CFU/g.

At the end of the storage period, the cell reduction was recorded as 1.79, 1.97, 0.46 and 1.62 log CFU/g decrease for *S. thermophilus* TH982 cells encapsulated with S, S+I, S+G, S+F, respectively.

From the data shown in Figure 3.2, the free cells (TH982) showed the highest viability during the storage period. In this regard, after 21 days, the analysis of viable cells of free TH982 revealed no significative differences between initial and final numbers (8.90 ± 0.08 log CFU/ml at initial time and 8.94 ± 0.05 log CFU/ml after 21 days). Also, gelatin as a co-encapsulating agent indicated that after 21 days the survival of cells was comparable to that of non-encapsulated ones and statistically different ($P < 0{,}0001$) to the control (sodium alginate alone), achieving a final number of viable bacteria of 8.04 ± 0.09 log CFU/g (initial number 8.50 ± 0.04 log CFU/g of capsules).

Notwithstanding, the other types of encapsulating matrices displayed a decrease in the number of viable cells after 21 days. Capsules of sodium alginate alone and in combination with the two prebiotics (inulin and 2'-FL) revealed a similar drop in viable bacteria counts; in particular a final count of 6.82 ± 0.19 log CFU/g of sodium alginate capsules (initial number of 8.61 ± 0.19 log CFU/g),

7.07 ± 0.20 log CFU/g of sodium alginate with inulin capsules (initial number of 9.04 ± 0.12 log CFU/g) and 7.35 ± 0.02 log CFU/g of sodium alginate with 2'-FL capsules (initial number of 8.97 ± 0.07 log CFU/g) were detected.

This result is in contrast with the majority of the studies performed to evaluate the survival of encapsulated bacteria under refrigerated conditions. For instance, encapsulation improved the stability of *L. plantarum* during storage (Wang et al., 2016), and encapsulation of bacteria in alginate was found to improve survival rates by one log when compared to free cell counts when stored in skimmed milk for 24 h (Rokka and Rantamäki, 2010).

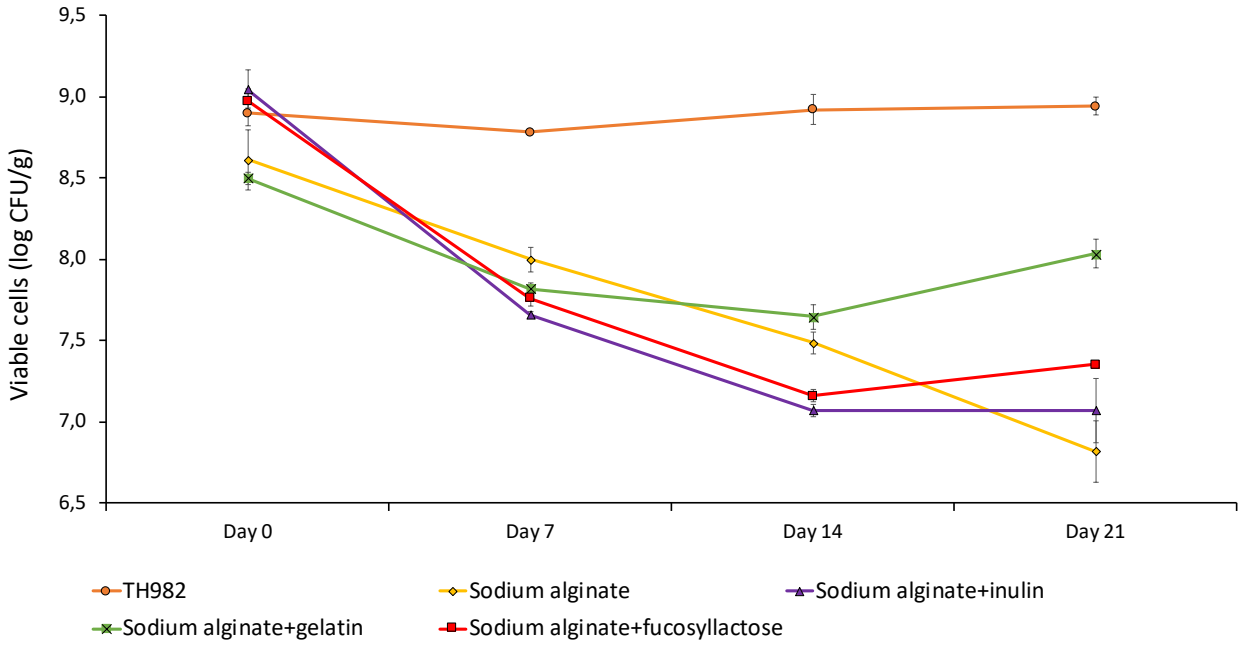


Figure 3.2 - Survivability of *S. thermophilus* TH982 encapsulated and free cells during 21 days storage in skimmed milk

Values shown are means ± standard deviations (n = 3)

Since FAO and International Dairy Federation (IDF) have recommended that the least viability of probiotics should be 10^7 CFU/g of product at the time of consumption (Shiby and Mishra, 2013), the four encapsulating materials that were used in this study were efficient in maintaining the required viability of *S. thermophilus* after refrigerated storage in skimmed milk. The high numbers suggested by IDF have been proposed to compensate for the possible reduction in the numbers of the probiotic organisms during passage through the stomach and the intestine.

It is important that the cells remain viable throughout the projected shelf-life of a product so that when consumed, the product contains sufficient viable cells.

The viability of probiotic bacteria in food products is affected by many intrinsic and extrinsic aspects such as dissolved oxygen and oxygen permeation through the package, post acidification in fermented products (lactic and acetic acids), pH, storage and incubation temperature, duration of fermentation, production of hydrogen peroxide due to bacterial metabolism, and processing conditions (Capela et al., 2006; Shah, 2000). Moreover, the specific strains used, the interaction between species (proto-cooperation), availability of nutrients, and the presence of growth promoters and inhibitors, can affect the survivability of viable cells.

The great survivability of free cells in skimmed milk is probably due to the fact that *S. thermophilus* is highly adapted to grow on lactose as energy source, which is transported by lactose permease (LacS) and, once entered the cell, is hydrolyzed into glucose and galactose by β-galactosidase (LacZ) (Hols et al., 2005). Hence, the entrapment of TH982 into capsules of different matrices has probably led to reduction of diffusion of lactose through the capsule layer or membrane limiting the possibility to use a carbon source.

Considering that viability is affected by cell concentration (Shah, 2000), it can be hypothesized that bacterial cells entrapped in a capsule represent a microenvironment with a high cell concentration.

The addition of glucose oxidase in yogurt processing has been reported as a potential technological solution to reduce the dissolved oxygen, thus minimizing the oxidative stress and positively affect probiotics growth, which have anaerobic characteristics (Batista et al., 2015). Therefore, to positively affect probiotics growth during storage, which can subsequently increase the number of bacteria to reach the gut, one possible solution can be represented by the addition of glucose oxidase to food products (Batista et al., 2015), thus inclusion of these enzymes in the encapsulating matrix could be considered.

3.4 Survival under simulated gastrointestinal conditions

Probiotic microorganisms must be resistant to gastric juices and be able to grow in the presence of bile salts or must be inserted in a food vehicle that allows them to survive passage through the stomach and exposure to bile (FAO/WHO, 2006).

The survival of probiotics in the gastrointestinal environment is a major limitation for their effectiveness, and microencapsulation represents a method to provide significant protection. The survival of free and encapsulated probiotic *S. thermophilus* TH982 during 1, 2, and 3 h of incubation in simulated gastrointestinal conditions is presented in Figure 3.3.

After 1 h of gastric juice, the highest reduction of viable cells was found, as aspected, for free TH982, as the initial number of 10.40 ± 0.10 log CFU/ml was diminished by 0.95 log CFU/ml. A notable

decrease in viability after 3 h of gastrointestinal conditions was confirmed for free TH982 cells, that diminished by approximately 1.60 log CFU/ml, dropping to 8.79 ± 0.02 log CFU/ml.

By contrast, for encapsulated cells with alginate alone, only 1.05 log CFU/g reduction of viable cells was observed, implying that microencapsulation provided effective protection compared to the free cells, as reported by Liao et al. (2019). This protection offered by sodium alginate might be related to the establishment of a hydrogel barrier through an external layer of sodium alginate that retarded the permeation of juices into the capsules and thus interaction with the probiotic cells (Jiménez-Pranteda et al., 2012). From a structural perspective, it might be explained by the acid-induced water loss of alginate in low pH. When pH is below the pKa values of carboxyl functions, alginate gets converted into alginic acid gel structures by H-bonds with the release of calcium ions resulting in a more dense gel due to water loss (Doumèche et al., 2004).

According to Sun and Griffiths (2000) the low pH conditions of the stomach (less than 2) resulted in dramatic decrease in the viability of *Bifidobacterium* strains. However, after microencapsulation of cells, the death rate was around 0.67% under the same conditions. When placed into simulated gastric fluid, free cells were exposed to the low pH immediately, while capsules might provide protection: the bacteria cells in the core and the surface of the capsules were subjected to a different pH. This pH gradient in capsules was dependent on the bead size and the exposure time in simulated gastric juice (Sun and Griffiths, 2000).

Capsules consisting of sodium alginate and gelatin showed low survivability during gastric environment; indeed the reduction of viable cells was higher (0.52 log CFU/g decrease) than the loss of encapsulated cells with inulin (no reduction detected) after 1 h of gastric condition (pH 2.5). This fact might be due to a variation of pH, which changes the gelatin charge with the amino and carboxyl groups, so the modification of cross-links and the structure of the chains could influence the swelling behavior of gelatin capsules (Duconseille et al., 2015).

An important result was given by the two types of prebiotics used as a co-encapsulating agent with sodium alginate: inulin and 2'-FL. After 1h of exposure to gastric conditions, only inulin indicated significant protection compared to the other agents (no reduction detected). However, after gastrointestinal incubation, 2'-FL and inulin revealed the highest cell survivability, leading to a very low, not significant, cell reduction (less than 0.45 log CFU/g decrease for both agents).

The decrease in the viability of free bacteria during gastric environment transit (0.95 log CFU/ml decrease) was also higher than that observed during 3h gastrointestinal (0.69 log CFU/ml decrease) incubation. In contrast, encapsulated bacteria experienced a greater reduction during gastrointestinal transit rather than in gastric conditions alone. This behavior may be due to the fact that alginate is stable in low-pH solutions: the ionotropic alginate gel formed by Ca^{2+} cross-linking of carboxylate

groups is insoluble at low pH, but exposure to neutral or alkaline pH solubilize the alginate, causing swelling (Gandomi et al., 2016).

As soon as the probiotic cells encapsulated leave the stomach (pH 2.5), the low pH around the beads is neutralized by the intestinal juice (pH 7.5), and as a consequence, the capsules are softened at higher pH and might be broken by the peristalsis of the small intestine, causing the released of bacteria cells from the capsules (Sun and Griffiths, 2000).

Regarding the gastric fluid pH, it should be noted that in the literature the values vary between 1 and 3. This pH range covers the values generally observed in the human stomach. Specifically, pepsin is often used as a gastric enzyme model, and generally, the average time to empty half of the stomach contents is 90 min (Sun and Griffiths, 2000). The intestinal pH values used in different studies are between 6.5 and 8. These values reflect the pH usually met in the gut (Gbassi and Vandamme, 2012).

Concerning the exposure time, it has to be noted that the gastric transit rate of both liquids and solids is significantly slower in women than in men, while ageing accelerates the small intestinal transit significantly in both gender. Therefore, gender and age differences have to be considered when performing simulated gastrointestinal transit (Graff et al., 2001).

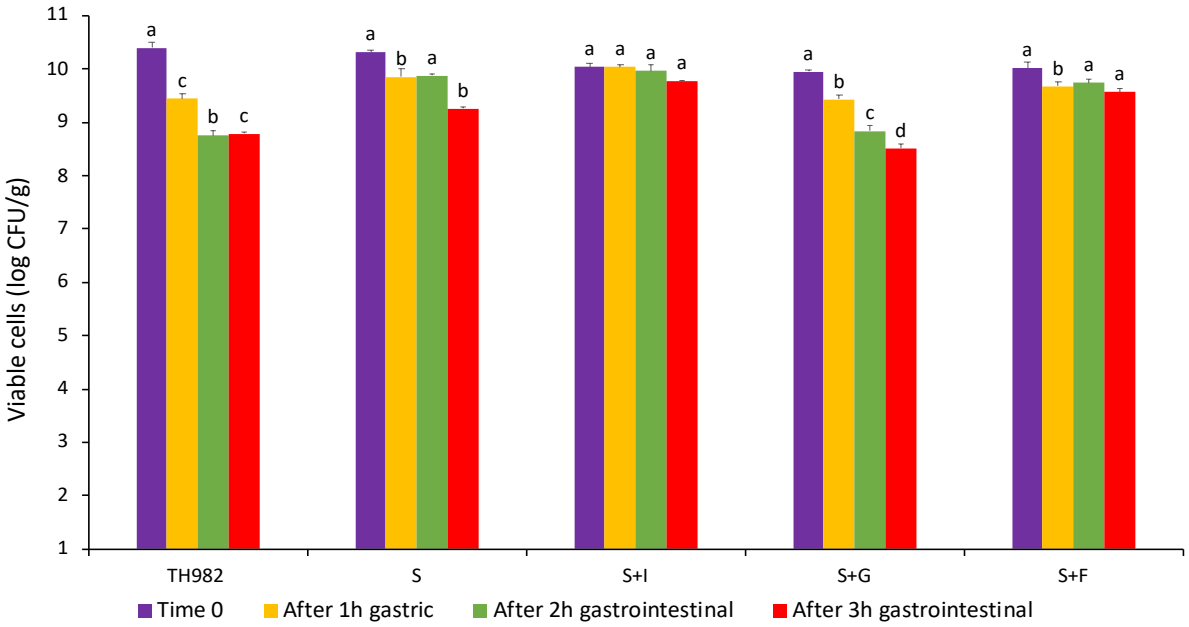


Figure 3.3 - Survivability of *S. thermophilus* TH982 encapsulated and free cells during exposure to *in vitro* simulated gastrointestinal conditions.

Superscript letters indicate statistically significant ($P < 0.05$) differences between different type of encapsulating matrix.

Values shown are means + standard deviations (n = 3)

The results of this investigation are in agreement with other studies. As Sultana et al. (2000) reported, the co-encapsulation with alginate and prebiotic (resistant starch) demonstrated synergic interaction in gelling and, as a result, may help to provide additional protection to the entrapped bacterial cells.
Another study indicates that encapsulation of probiotic *L. rhamnosus* in chitosan-coated alginate bead exhibited a higher survival rate during gastrointestinal treatments compared to free bacteria, and inulin demonstrated a fortification effect, improving the viability of encapsulated bacteria during product storage as well as during GIT transit (Gandomi et al., 2016).
The combination of calcium alginate with prebiotics such as inulin improves the viability of probiotics and facilitates the formation of integrated structure of capsules. Researchers found better protection of cells in coated capsules after simulated gastric juice of *L. casei* and *B. bifidum*, deducing that it might be attributed to the addition of inulin, which acts as a prebiotic (Khosravi Zanjani et al., 2014).
Sathyabama et al. (2014) tested bacterial growth on different potential prebiotics (sugar beet, chicory, and oats) to select the co-encapsulating agent to be used with alginate. The variation in the oligosaccharide composition of prebiotic sources might be the reason for the difference in the growth rate. The rate at which oligosaccharides are fermented depends on the degree of polymerization, glycosidic linkage, degree of branching, and synergy between bacteria during fermentation. They found a significant difference among the prebiotics tested on bacterial growth: sugar beet and chicory were found to be an efficient prebiotic with high fermentation rate and potent in co-encapsulation with probiotics in alginate matrix.
Prebiotics, such as 2'-FL and inulin, which could provide good protection and even promote cell proliferation, appeared to contribute to the growth of *S. thermophilus* TH982. In addition, oligosaccharides are difficult to be decomposed by enzymes in digestive juice but can be metabolized by beneficial bacteria in the colon (Liao et al., 2019). It has been further demonstrated that oligosaccharides contained in human milk have an extraordinary resistance to hydrolysis by digestive enzymes of the small intestine (Engfer et al., 2000).
Therefore, besides the protective function, oligosaccharides entrapped in the capsule could also provide selective carbon sources for probiotics and even promote the proliferation of some strains.
A choice of a microencapsulation system has to consider the best suitable technology to preserve and even promote the functionality of probiotics. One "gentle" approach for encapsulation is the extrusion technique in combination with matrix molecules (Nedovic et al., 2011). Alginate is the most widely used encapsulating material, thanks to its low cost, ease of handling, and biocompatibility (Krasaekoopt et al., 2004). According to Jiménez-Pranteda et al. (2012), *L. plantarum* encapsulated with jamilan:gellan gum (1%:1%) after 2 h of simulated gastrointestinal juice showed a lack of

protection, while *L. rhamnosus* demonstrated a minor decrease. This behavior demonstrated that the biocompatibility of certain encapsulating polymers with specific bacteria is of great importance and may even differ among strains of the same probiotic species (Rodrigues et al., 2011).

Annan et al. (2008) noted that the simulation of gastric juices for *in vitro* studies tends to overestimate viability losses that would occur *in vivo*: during and after a meal, the buffering capacity of foods may temporarily elevate the gastric pH so that the cells can reach their site of action in sufficiently high numbers to produce a physiological effect (Mainville et al., 2005). As a confirmation, non-protected cells consumed in a dried form have lower recovery levels in stools than those consumed in milk or cheese (Champagne and Fustier, 2007).

From this study, it is possible to conclude that the most advantageous encapsulating agents for the transit in the gastrointestinal tract are prebiotics: inulin and 2'-FL (0.28 log CFU/g and 0.44 log CFU/g decrease respectively from the initial number of viable cells).

Furthermore, considering the storage period, using these two types of matrices, the reduction that occurs after 21 days at 4 °C in skimmed milk was 1.97 log CFU/g for inulin and 1.62 log CFU/g for 2'-FL (not statistically different). It can be concluded that, if the number of cells in the food product is equal or higher to that required at consumption time (10^7 CFU/g), the sodium alginate-based microencapsulation with 2% of inulin or 2% of 2'-FL might maintain a required number of viable cells after the gastrointestinal passage.

3.5 Release kinetics

To evaluate the survival under gastrointestinal conditions, it is essential to ensure that the capsules give protection through the simulated gastrointestinal passage, and ensure that the encapsulation matrix allows a release of viable and metabolically active cells in the intestine (Gbassi and Vandamme, 2012). The use of capsules minimizes the bactericidal effects of the stomach environment and maximizes the number of probiotic cells reaching the ileum.

After passing through the stomach, microcapsules will reach the intestine with higher pH and release the probiotic cells (Shi et al., 2013b). The release of cells from microcapsules in the colon is essential for the growth and colonization of probiotics. In the absence of this event, the organisms in the beads will be washed out from the body without exerting any beneficial effect (Sabikhi et al., 2010). The release can be time-, site-, rate-, stimulus-specific or triggered by changes in pH, temperature, irradiation, or osmotic shock. The steps involved in the release of an active agent (bacteria cells) that is dissolved in the matrix are (Pothakamury and Barbosa-Cánovas, 1995): A) diffusion of the active agent to the surface of the matrix; B) partition of the active agent between the matrix and the elution

medium (i.e., the surrounding food or intestinal environment); C) transport away from the matrix surface.

The release of encapsulated *S. thermophilus* TH982 after incubation in simulated intestinal fluid (pH 7.5) was evaluated at 30, 60, 90, 120, 150, and 180 min, as reported in Figure 3.4. The release characteristics have been evaluated at a predetermined time point, every 30 min up to 180 min in a simulated intestinal fluid as reported by Shi et al. (2013a).

Just after 30 min of agitation (100 rpm) at 37 °C in K_2HPO_4, a quick release of cells from sodium alginate based encapsulation with the prebiotic 2'-FL was detected. Similarly, cells encapsulated and co-encapsulated with different types of oligosaccharides started to be slowly released after 30 min into the simulated intestinal fluid (pH 7.4) (Liao et al., 2019), reaching a plateau (Figure 3.4). Considering the initial number of cells entrapped in this type of capsules (9.73 ± 0.04 log CFU/g of capsule), the count of cells released in the simulated intestinal fluid after 30 min was 8.83 ± 0.04 log CFU/ml, representing the highest value even in comparison with the release of other types of encapsulating matrices after 180 min. This result is a confirmation of what was observed while performing the experiment: after 30 min, there was a visible dissolution and solubilization of 2'-FL capsules, behavior not found in other capsules. Moreover, the survival of released cells suggests that once liberated, *S. thermophilus* TH982 is able to withstand the simulated intestinal conditions at pH 7.5; as evidence, after 180 min, the count of cells released from capsules with 2'-FL was 9.72 ± 0.05 log CFU/ml, which means 99.89% of initial entrapped cells, representing a result not reported in other studies in the literature up to date. An efficient release of viable and metabolically active cells in the intestine is one of the microencapsulation aims (Mandal et al., 2006).

While the release profile indicated that alginate-based encapsulation with 2'-FL had fast release property in intestinal fluid, the ability of sodium alginate (initial count 9.43 ± 0.04 log CFU/g), sodium alginate with gelatin (initial number 9.62 ± 0.03 log CFU/g), and sodium alginate with inulin (initial count 9.68 ± 0.03 log CFU/g) to release the entrapped cells is lower.

After 30 and 90 min, the relase profile was similar in capsules with sodium alginate alone (6.54 ± 0.04 log CFU/ml released after 30 min and 7.07 ± 0.11 log CFU/ml released after 90 min) and in combination with gelatin (6.52 ± 0.06 log CFU/ml released after 30 min and 6.91 ± 0.19 log CFU/ml released after 90 min). However, after 180 min of exposure, co-encapsulation with the fructan polysaccharide (inulin) (initial count 9.68 ± 0.03 log CFU/g of capsules) and protein (gelatin) (initial count 9.62 ± 0.03 log CFU/g of capsules) demonstrated a comparable release, 7.35 ± 0.14 log CFU/ml and 7.15 ± 0.10 log CFU/ml, respectively. This result is quite unexpected because during simulation of passage in the gastrointestinal tract, inulin showed a behavior similar to 2'-FL. Thus, there was a significant difference between the release kinetic of the two prebiotics, inulin and 2'-FL ($P < 0.0001$),

at the end of incubation time, showing a difference of 2.37 log CFU/ml in the number of released cells.

The ability to release the cells from capsules of sodium alginate and gelatin was the lowest detected (7.15 ± 0.10 log CFU/ml). This result is in accordance with Li et al. (2009) which reported that the incorporation of gelatin as a co-encapsulating agent with alginate did not affect the kinetics of release from capsules.

The second highest release of entrapped cells was observed in capsules of sodium alginate alone, specifically 7.45 ± 0.10 log CFU/ml released at the end of the incubation (180 min), while inulin showed a low capacity of releasing probiotic cells in the small intestine.

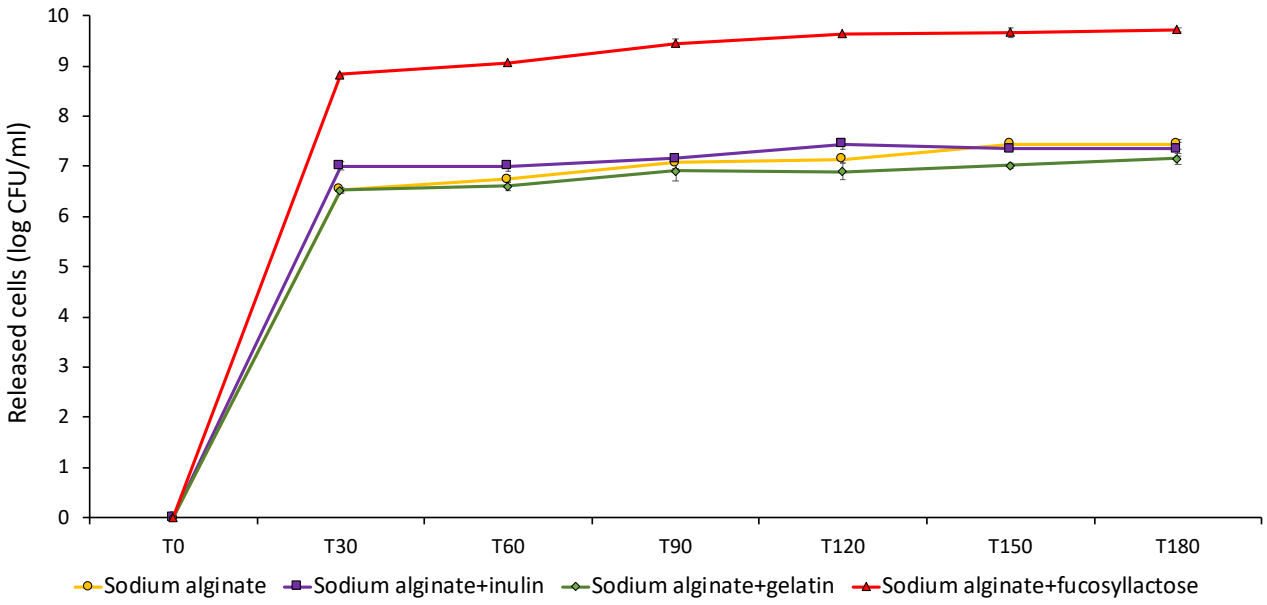


Figure 3.4 - Release of entrapped cells of *S. thermophilus* TH982 in simulated intestinal environment.

Values shown are means ± standard deviations (n = 3)

The release of the cells from microcapsules in the colon is essential for the growth and colonization of probiotics (Chen et al., 2014). When the capsules are opened in the small intestine, the released cells are expected to arrive in the large intestine (Mortazavian et al., 2007). Iyer et al. (2004), investigated the survival and the subsequent release of alginate capsules in samples of different sections of the gastrointestinal tract (stomach, duodenum, anterior small intestine, posterior small intestine, and colon). It was demonstrated that the principal site of bacterial release in the gut was the small intestine (pH 6.5-6.8); thus, the majority of cells were released in both anterior and posterior ileal contents in 1 h. This represents a significant result because many important functions attributed to probiotics rely on the presence of the selected bacteria in the small bowel. For example, this

segment of the gut is important for the presence of Peyer's patches and other gut-associated lymphoid structures (GALTs) that are thought to play a critical role in immunomodulation (Iyer et al., 2004).

Many studies investigated the release of encapsulated probiotics in simulated intestinal juice. Similar to our observations, Sabikhi et al. (2010) reported the release of encapsulated *L. acidophilus* in alginate-starch microspheres using simulated colonic juice with the same formulation (KH_2PO_4, pH 7.4 ± 0.2 during 2.5 h). They found that cells count after 150 min of incubation was 7.45 log CFU/ml, suggesting that all the microencapsulated cells were released at this time (initial number of 7.47 log CFU/g of capsule). Although 150 min is approx the same time needed for intestinal transit of microflora, they did not found a significant release after 30 or 60 min.

Another study pointed out a progressive release of viable cells from whey-protein-based microcapsules in simulated intestinal conditions. The researchers attributed the considerable increase of 3.5 log in cell number after 1 h of exposure to simulated pancreatic juice not only to cell multiplication, but also to a massive release of uninjured bifidobacteria from degraded microcapsules and/or recovery of sublethally injured cells (Picot and Lacroix, 2004).

In a different study, *L. casei* was encapsulated in different alginate concentrations (2%, 3%, or 4%), and different parameters were evaluated. The survival of entrapped cells in low pH and high bile salt concentration increased proportionately with increasing alginate concentrations. However, the release of bacteria from capsules in simulated colonic pH solution (KH_2PO_4, pH 7.4 ± 0.2) was not affected by different alginate concentrations after 60 min of exposure (Mandal et al., 2006). On the other hand, a significantly decrease of the release rate of encapsulated probiotic bacteria from microspheres was detected with the increase of alginate concentrations in alginate-milk microspheres (Shi et al., 2013a).

An important aspect when performing microencapsulation of probiotics to improve survival in the gastrointestinal tract is the definition of the physicochemical characteristics of the encapsulating materials in order to predict their mechanisms of disintegration or dissolution under varying conditions of pH and salinity and their interactions with probiotic cells or other components present in the digestive tract (Gbassi and Vandamme, 2012). As an example, Chen et al. (2014) found that the dissociation of alginate-based gel network is due to electrostatic repulsion between whey proteins, which are negatively charged, and the carboxyl group from the alginate molecules. Moreover, it is well known that alginate gel formed through calcium ions is very rigid and only swells slowly at neutral or basic pH, allowing subsequent release in the basic environment of intestinal fluids (Sathyabama et al., 2014).

A fully release of *L. plantarum* encapsulated with sodium alginate and sodium alginate with inulin in 60 min of exposure into simulated intestinal fluid has been reported. From the results, it was concluded that the release mechanism was probably due to the replacement of calcium ion in the

encapsulation matrices. Also, capsules with inulin showed a faster release rate during the first 20 min which could have been induced by the addition of inulin in sodium alginate affecting the binding of calcium ion (Wang et al., 2016).

The above-mentioned study indicated a mechanism that may be applied to explain the fast and fully released of TH982 ecapsulated with sodium alginate and 2'-FL. This small soluble milk glycan used as encapsulating agent in this experiment is formed by fucose linked to the 2 position of β-Gal residues of lactose. 2'-FL is considered a neutral HMO (Engfer et al., 2000), hence no electrostatic repulsions may occur with the negatively charged carboxyl groups of sodium alginate structure. Therefore, the complete release of the cells is probably due to the vigorous interaction of the prebiotic structure with the divalent cations (Ca^{2+}) of the sodium alginate network, resulting in a disintegration of the "egg box structure", as observed after 30 min of incubation in K_2HPO_4. This process is well known and recognized to explain the inulin capsules releasing behavior in simulated intestinal fluid, a prebiotic commonly used as encapsulating material. However, this represents the first evidence of the higher ability of capsules created with a novel molecule (2'-FL), to release active cells in the intestinal target, even in comparison to the extensively studied molecule inulin.

4 CONCLUSIONS

The present study compared the ability of three different alginate-based microcapsules to increase the survival of the potential probiotic strain *S. thermophilus* TH982 in a simulated gastrointestinal environment.

The capsules were successfully obtained by the extrusion technique, as demonstrated from the high capacity of entrapment, from 90.50% to 98.61%, found in all the different materials used.

The outcomes obtained after 21 days of storage of the capsules in skimmed milk indicated that microencapsulation might not be a promising method to enhance stability of viable cells at 4 °C, except for gelatin. This could suggests that one possible solution to achieve a considerable amount of viable cells after storage period, is to consider the addition of bacteria non-encapsulated and microencapsulated in the same food product, or to combine gelatin with a molecule, such as 2'-FL, which can allow good resistance and released of cells once ingested.

Interesting findings were obtained after the exposure to a simulated gastrointestinal environment: after 3 h of incubation the two prebiotic molecules used (inulin and 2'-FL) revealed a good ability to improve protection of TH982, ensuring survival in the harsh acidic conditions and bile concentration, as demonstrated by the low reduction of viable cells obtained (0.28 log CFU/g decrease and 0.44 log CFU/g decrase, respectively).

Nevertheless, after further investigation, only the human milk oligosaccharide prebiotic agent (2'-FL) showed the capacity to release nearly all the TH982 cells encapsulated in simulated intestinal juice (pH 7.5). Considering that Human Milk Oligosaccharides are resistant to the low gastric pH and enzymatic digestion in the upper gastrointestinal tract, as confirmed in this study, 2'-FL might reach the lower gastrointestinal tract intact where it provides nutrients for the colonic microbiota. In view of the great release attitude in the intestinal environment, it is possible to conclude that 2'-FL is the most suitable encapsulating agent for *S. thermophilus* TH982 .

The present work represents the first example of microencapsulation with this novel molecule, suggesting that further studied should be of interest to investigate more in details the prebiotic properties as encapsulating agent. In particular, different concentrations of 2'-FL and/or co-encapsulation with other prebiotics should be investigated and compared to optimize the encapsulation technology and to obtain the best combination.

The promising results obtained with this work have been obtained using *in vitro* tests, hence future research should be performed *in vivo* to assess the mainteinment of this behaviour.

Finally, considering that capsules containing probiotic cells are designed to be incorporated into foods as vehicle (e.g., yogurt), specific morphological studies, including a psychochemical analysis, along with a sensory evaluation should be taken in to account.

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