Hungarian University of Agriculture and Life Sciences

EVALUATION OF DIFFERENT MICROENCAPSULATION TECHNIQUES FOR IMPROVING THE FUNCTIONAL EFFECTIVENESS OF PROBIOTIC-CONTAINING FOODS

DOI: 10.54598/001630

Linh Phuong Ta

Budapest
2021

The PhD School

Name: Doctoral School of Food Science
Discipline: Food Science
Head: **Prof. Dr. Livia Simon-Sarkadi**
Professor, DSc
Hungarian University of Agriculture and Life Sciences
Institute of Food Science and Technology
Department of Applied Chemistry

Supervisors: **Dr. Erika Bujna**
Associate professor, PhD
Hungarian University of Agriculture and Life Sciences
Institute of Food Science and Technology
Department of Bioengineering and Alcoholic Drink Technology

Dr. Szilárd Kun
Associate professor, PhD
Hungarian University of Agriculture and Life Sciences
Institute of Food Science and Technology
Department of Bioengineering and Alcoholic Drink Technology

Approval signatures of the Head of Doctoral School and the Supervisors:

The candidate has fulfilled all the conditions prescribed by the Doctoral School of Hungarian University of Agriculture and Life Sciences, the comments and suggestion at the thesis workshop were taken into consideration when revising the thesis, so the dissertation can be submitted to a public debate.

.....	.....
Approval of the Head of Doctoral School	Approval of the Supervisors

Table of Contents

ABBREVIATIONS

Capsule material (capsule type)

ALG	Alginate (capsules)
STA-ALG	Resistant starch - Alginate (capsules)
LAC-ALG	Lactulose - Alginate (capsules)
LS40L-ALG	Lactosucrose LS40L- alginate (capsules)
LS55L-ALG	Lactosucrose LS55L- alginate (capsules)
CHI coat. ALG	Chitosan coated alginate (capsules)
SDEX coat ALG	DEAE Sephadex A 50- coated alginate (capsules)
GEL-XNT	Gellan gum - Xanthan gum (capsules)
CAR-LBG	κ-carrageenan - Locust bean gum (capsules)
CHI	Chitosan (capsules)

Bacterial genera

L.	*Lactobacillus*
B.	*Bifidobacterium*

Other

FAO	Food and Agricultural Organization
WHO	World Health Organization
SCFA	Short-chain fatty acid
CFU	Colony-forming unit
Log CFU	CFU expressed in logarithmic (base 10) unit
SSF	Simulated salivary fluid
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
FITC	Fluorescein isothiocyanate
Na-Fluo	Sodium fluorescein
LbL	Layer-by-layer
CMC	Carboxymethyl cellulose
CHI	Chitosan (polymer)
D (4,3)	Volume-weighted mean diameter
Span	Width of the size distribution

1 INTRODUCTION

During the last decades there has been a considerable demand for various foods that can contribute to health and overall well-being. Growing evidence indicate that intake of foods fortified with probiotics, namely probiotic foods can potentially confer numerous benefits to human health, which in turn has established a big market of these foods worldwide. To date, different dairy products have served as traditional forms of probiotic consumption. Although, due to the high prevalence of people with lactose intolerance, milk protein allergy, hypercholesterolemia, calorie concerns and strict vegetarian dietary patterns in these days, more preference has been directed towards choosing non-dairy, particularly plant-based products. However, there are several technological challenges that need to be addressed when it comes to developing probiotic products, particularly of plant-based types. In specific, these probiotics have to confront a variety of stress factors during food processing (e.g. heat treatment), food storage (e.g. acidic conditions of some plant-based products) and subsequent gastrointestinal transit (e.g., gastric acid, bile salt), by which their health effects cannot be realised as effectively as desired. This, in turn, can question the claimed functionality of the probiotic products as well.

Microencapsulation of probiotics in a protective polymer matrix or with polymer coating is one of the recent potential approaches to protect the viability of probiotics under several harsh conditions, and also effectively deliver them to their therapeutic sites of action within the gastrointestinal tract, along with targeted release. Several delivery (capsule) systems for probiotics have been developed in previous studies to date. However, the main problem with them is their limited adaptability for food industrial and commercial applications, considering the scale-up difficulties of their microencapsulation processes, too large capsules for incorporating in food products, or insufficient probiotic protection against strong acidic and bile salt conditions, among others. Furthermore, the effect of these probiotic-loaded capsules in real food matrices, especially of plant-based types, has been insufficiently assessed so far. Last but not least, mucoadhesion aspect of these capsules – also necessary for more effective gastrointestinal delivery (and release) of encapsulated probiotics – has particularly not been reported in previous studies.

In light of above-mentioned concerns, the objective of my PhD work is to find the most promising encapsulation materials and techniques for development of microcapsule systems that can be utilised for effective protection and gastrointestinal delivery of probiotics, and at the same time, can be well-

adapted for food industrial applications, thereby for developing novel probiotic non-dairy food products. In order to solve these research problems, the following tasks were set to accomplish:

- Formation of different probiotic-loaded capsule systems by applying different materials for microencapsulation process, including prebiotics such as resistant starch, lactulose and lactosucrose; hydrocolloid polymers such as alginate, gellan gum, xanthan gum, κ-carrageenan, locust bean gum, carboxymethyl cellulose and chitosan. In addition, polymer coating of bacteria-loaded alginate capsules with either chitosan or DEAE Sephadex was also aimed to perform.
- Encapsulation of a model probiotic strain with different chosen techniques, including the two most commonly reported techniques of extrusion and emulsification (external gelation-involving type), and the two less commonly studied ones of electrospraying and layer-by-layer self-assembly techniques.
- Evaluation and comparison of differently formed probiotic-loaded capsule systems – regarding encapsulation material and technique – for their efficacy in probiotic delivery, based on the following physical and physiological aspects:
    - Capsule size and size distribution
    - Encapsulation efficiency of viable probiotics
    - Viability of encapsulated probiotics under commonly applied *in vitro* gastric and/or intestinal conditions.
        - Viability of encapsulated probiotics under *in vitro* digestion conditions based on a standardised Infogest protocol.
    - Heat tolerance of encapsulated probiotics.
    - Long-term storage stability and metabolic activity of encapsulated probiotics in different commercial plant-based beverages at different temperatures.
    - Comparison of the encapsulated probiotics with unencapsulated probiotics in terms of their survival rate under the above-mentioned stress conditions.
    - Mucoadhesion property of capsule systems.
- Comparison of encapsulation effect on the physiological activities (i.e., survival rate under stress conditions) of probiotic *Lactobacillus* and *Bifidobacterium* strains

2 LITERATURE REVIEW

2.1 PROBIOTIC FOODS

It has been a long time that functional foods have exploded into the mainstream thanks to the increasing trend of health awareness worldwide. According to a very commonly used description suggested by the European consensus, a food can be considered as functional if its consumption not only covers the essential nutrition and energy needs, but also offers some additional therapeutic effects on one or more target functions in the human body, by which it can contribute to improved health and well-being and/or the reduction of risk of disease (Ashaolu, 2020; Lobo et al., 2010; Quiroz-Iturra et al., 2017; Roberfroid, 2000). The functionalisation of foods can be derived by several approaches, including the addition or the enhancement of certain beneficial bioactive agents. For examples, functional foods supplied with probiotic microorganisms, namely probiotic foods have attracted considerable attention of consumers for their claimed promoting effect on the healthy balance of human gut microflora (Martins et al., 2013). In fact, these products account for by far the greatest growing branch of the whole functional food market these days (Aspri et al., 2020). According to a recent research report (Probiotics Market, 2019), the commercial value of this food category reached around USD 49.4 Billion (~ EUR 41.8 Billion) in 2018 and is predicted to worth around USD 69.3 Billion (~ EUR 58.7 Billion) by 2023.

Dairy products, such as yogurt, cheese, sour milk drink, and kefir, serve as the most conventional and common forms of probiotic consumption, as they can provide well-established matrices for these probiotics to integrate through fermentation (Aspri et al., 2020; De Prisco & Mauriello, 2016; Martins et al., 2013; Tripathi & Giri, 2014). However, the consumption of dairy-based probiotic products can cause health risks for a large number of those who suffer from lactose intolerance, milk protein allergy, or is just not preferable for those who follow strict vegetarian lifestyle (Bayless et al., 2017; Gawkowski & Chikindas, 2013; Martins et al., 2013; Sethi et al., 2016; Szilagyi & Ishayek, 2018). Besides, depending on the animal origin of milk, high fat and cholesterol content may be found in dairy products, by which their excessive consumption can increase the total and LDL-cholesterol level in the blood (Kumar et al., 2015). Therefore, in light of these concerns, a wide range of alternative probiotic sources should be offered in a hope of expanding their consumer base.

Non-dairy probiotic food products

In recent times, several studies have reported that fruit, vegetable and cereal-based beverages, among others, can represent potential matrices for developing non-dairy probiotic products, due to their high

sugar and other nutrient contents essential for probiotic growth (Aspri et al., 2020; De Prisco & Mauriello, 2016; Kandylis et al., 2016; Valero-Cases et al., 2020). Although, there are some risk factors that may make the use of these plant-based matrices challenging for such purpose (see Section 2.4.1.2). To date, different fruit, vegetable, and cereal-based beverages have also been produced for commercial purposes, which examples are shown in Table 1. On commercial aspects, probiotic consumption in these specific forms is greatly ideal, considering that the conventional version of these plant-based foods and beverages are already perceived as healthy for providing high nutritional values like vitamins, minerals, trace elements, phytochemicals and dietary fibres (Dey, 2018; Fardet, 2017; Kandylis et al., 2016), not to mention that their consumption can be a great choice for vegetarians and meat-eaters alike. Fruit and vegetable products are particularly favourable for naturally being lactose-free, allergen-free, low in fat and cholesterol content (Aspri et al., 2020; Butler & Fletcher, 2019; Gawkowski & Chikindas, 2013), and low-calorie which make their consumption ideal for people who are trying to lose or maintain weight to a healthy level (Slayton & Pfau, 2019).

Table 1. Some examples of commercially available probiotic-containing non-dairy beverages (Aspri et al., 2020)

Brand	Beverage type	Probiotic strains	Manufacturer
Avenly velle	Oat-based drink	*Lactobacillus* and *Bifidobacterium*	Avenly Oy Ltd., Finland
Biola	Fruit juice	*L. rhamnosus* GG	Tine BA, Norway
Bioprofit / Gefilus	Fruit juice	*L. rhamnosus* GG, *Probionibacterium freudenreichii*, *Shermanii* JS / *L. rhamnosus* GG	Valio Ltd., Finland
Bravo Friscus	Fruit juice	*L. plantarum* HEAL9, *L. paracasei* 8700:2	Skanemajerier, Sweden
GoodBelly	Fruit juice	*L. plantarum* 299v	NextFoods, USA
Healthy life probiotics	Fruit juice	*L. paracasei* 8700:2, *L. plantarum* Hea19	Golden circle, Australia
Mucilon	Oat and rice-based porridge for infants	*Bifidus* BL	Nestlé, Switzerland
ProViva	Fermented fruit drink with oatmeal	*L. plantarum* 299v	Skane Dairy, Sweden
Malee Probiotics	Fruit juices such prune, grape and orange	*L. paracasei*	Malee Enterprise Ltd., Thailand
Tropicana probiotics	Fruit juice mixtures such as strawberry and banana, pineapple and mango and peach passion fruit	*B. lactis*	Tropicana, USA

In specific, the consumption of **beetroot juice** has been reported to potentially modulate diabetes and insulin homeostasis, platelet aggregation, vascular and endothelial functions, reduce blood pressure, oxidative stress and inflammations, and also promote liver and renal health (Mirmiran et al., 2020;

Zamani et al., 2020) due to its high polyphenols, flavonoids, and nitrate content. Beetroot itself contains high amount of such phenolic acids as gallic, syringic, caffeic and ferulic acids, and also such natural red pigment as betalain (averagely 1103 ± 253 mg/L) (Wruss et al., 2015) which is considered as potent antioxidant, anti-inflammatory and chemo-preventive agent (Clifford et al., 2015). Beetroot juice is also a great sources of vitamin A, B_6, folate, and such minerals as calcium, magnesium, manganese, phosphorus, copper, zinc and iron (Eske & Marengo, 2019). **Oat drink** has been demonstrated to be one of the most promising functional plant-based beverages. For its high content of soluble fibres such as ß-glucan, oat milk can support the digestion process, the reduction in blood glucose level by delaying gastric emptying time, and also has been associated with hypocholesterolemic effect by lowering the total and the LDL cholesterol level (Sethi et al., 2016). Commercial oat milk is generally rich in vitamin A, D, B_2, B_{12} (through fortification), and minerals like copper, zinc, manganese, and magnesium (Purdie & Syn, 2020). In addition, the majority of fatty acids in oat milk are unsaturated unlike those in cow's milk (Önning et al., 1998), and also it contains exclusively polyphenols such as avenanthramides with antioxidant, anticancer antiatherogenic, and cardio protective activities (Marmouzi & Ezzat, 2018). Compared to other dairy milk substitutes including almond and rice drink, oat drink provides better protein intakes with good amino acid balance (Purdie & Syn, 2020) which would be greatly ideal for individuals on vegan diets.

2.2 PROBIOTIC MICROORGANISMS

The term 'probiotics' (originated from Latin, means 'for life') have been defined by several ways, among of which the most reported one is: 'Probiotics are such live microorganisms which when administered in adequate amounts confer health benefits on the host' (Fuller & Gibson, 1997; WHO/FAO, 2001).

The first use of 'probiotics' dates back to around 2000 BC when the positive effect of the spontaneous fermentation process was first discovered on the shelf-life of milk. Although, the existence of these microorganisms (including the name 'probiotics') itself was still not unknown at that time (Nakazawa & Hosono, 1992). In the early 1900s years, a French microbiologist, Louis Pasteur was the one who first identified that the process of fermentation is aroused from the physiological activity of the microorganisms (Gasbarrini et al., 2016). Besides, it was also him who made a first discovery on the lactic acid producing bacteria (Neubaier & Mollet, 2002). Later on, the concept of human health benefits of these microbes was first occurred to a Russian scientist, Elie Metchnikoff after recognising that the longer life span that once experienced for the young Bulgarian rural habitants is possibly

associated with the regular consumption of fermented dairy products containing *Lactobacillus* strains. He later also suggested that when lactic acid bacteria like lactobacilli are ingested with dairy products and successfully reach the colon, they might be capable of preventing the activity of toxin-producing and proteolytic bacteria that would be responsible for diseases and rapid ageing (Gasbarrini et al., 2016; Sousa e Silva & Freitas, 2014). In the beginning of 1930s, after isolating the first strains from the intestinal tract of healthy individuals and discovering their good tolerance of gastric and bile acid, Shirota tested and commercialised his first fermented dairy drink product named Shirota (later as *Lactobacillus casei* Shirota) under the company of Yakult Honsha (Amara & Shibl, 2015).

2.2.1 Criteria for the use of probiotics in foods and in humans

In order to apply microorganisms as probiotics in foods and for human dietary purposes, the following physiological, technological and safety-related criteria should be fulfilled by the candidate microorganisms (Frakolaki et al., 2020; Kumar & Salminen, 2016; Pech-Canul et al., 2020; Syngai et al., 2016; Tamime & Thomas, 2017; Zielińska et al., 2018):

Physiological criteria:

- have clinically verified positive effects on human health (e.g., stimulation of immune system without inflammatory impacts, inhibition of pathogen colonisation)
- be capable of maintaining their viability and their metabolic activity during the processing and the storage of the carrier food products, and during the subsequent passage through the gastrointestinal tract
- have an ability to adhere and colonise on the gastrointestinal mucosal barrier surface (at least temporarily)

Technological criteria:

- the large-scale production of the probiotic culture is technologically and reasonably feasible
- do not negatively alter the sensory quality of the food in which they are incorporated and stored
- be capable of maintaining or, if desired, improving the characteristics (e.g., viscosity, texture, etc) of the carrier foods
- show fermentation ability on the certain substrates while not inhibiting other beneficial strains (e.g. starter cultures) in the same food product

Safety criteria:

- be classified as generally recognised as safe (GRAS)
- have not been associated previously with the development of any diseases or disorders

- do not exhibit any pathogenic, virulence or any other toxic factors
- do not have any transmittable antibiotic resistance genes
- do not increase the permeability of gut mucosal barrier
- do exert antagonist effect on harmful microorganisms
- be preferably among the common inhabitants of the healthy human gastrointestinal tract

2.2.2 Overview of common probiotic strains

Probiotic functionality is often considered as a strain-specific microbial trait (not specified to either genus or species level), and on this aspect, the most reported probiotic strains belong to the genera *Lactobacillus* and *Bifidobacterium*, and *vice versa* (Cook et al., 2012; Gawkowski & Chikindas, 2013; Herbel et al., 2013; Li et al., 2019). Besides, they are also the most widely applied bacteria in modern probiotic products, not only for being associated with numerous health benefits, but also for their 'Generally Regarded as Safe' (GRAS) certification, having favourable metabolic and technological potentials, being dominant residents of the human gut microflora, and also for having the longest tradition in the food application (Saarela, 2017; Tripathi & Giri, 2014).

2.2.2.1 Lactobacillus

Genus *Lactobacillus (L.)* make up the largest part of the lactic acid bacteria. In terms of the latest taxonomical background, they belong to the family of *Lactobacillaceae* and to the order of *Lactobacillales*, within the class of *Bacilli* and the phylum of *Firmicutes* (Schoch et al., 2020; Zheng et al., 2020). Bacteria of this genus are characterised as Gram-positive, anaerobes or aerotolerant, catalase-negative, non-sporulating, non-motile and rod-shaped (Bratcher, 2018; Ibrahim & Ouwehand, 2019). Their optimal growth is generally induced at 30 - 40°C, at pH 6.5 and in the presence of complex nutrient sources (including vitamins, minerals, amino acids) (Terpou et al., 2019).They can ferment a large amount of sugars from their environment and as being lactic acid bacteria, convert them into lactic acid as a primary metabolite. Depending on the specific strain, they can exhibit this lactic acid production through obligate homofermentative (represented by most *Lactobacillus*, including *L. acidophilus, L. delbrueckii* subsp. *bulgaricus, L. gasseri, L. helveticus, L. jensenii, L. salivarius*), obligate heterofermentative (e.g., *L. brevis, L. fermentum, L. reuteri*) or facultative heterofermentative (represented by e.g., *L. casei* and *L. plantarum)* metabolic pathways (Boonma et al., 2015; Ibrahim & Ouwehand, 2019). It has been reported that around 85-90% of these sugars in the fermentation medium become lactate via homofermentative conversion (Siegrist, 2013). The uptake of the external free sugars is assured through either their specific permease or their specific phosphotransferase system (PTS). The sugar adaptability is largely determined by the complexity of

the transport systems which can vary with the strains. Fewer, but still some amount of amino acid is also utilised by lactobacilli for their growth, for which they apply their multi-step proteolytic system (von Wright & Axelsson, 2011).

Lactobacilli are one of the most common strains used in both the traditional and modern production and the preservation of different foods, including milk (e.g yogurt, cheese, kefir, fermented milk, kumis), meat (e.g. sausage), vegetables (e.g., sauerkraut, pickles) (Ibrahim & Ouwehand, 2019; Kumar & Salminen, 2016; Muriana & Luchansky, 1993).

Lactobacillus casei

Bacteria belonging to the species *L. casei* are typically isolated from milk and dairy products, but also from fermented sausage, fruits, vegetables, wine, silage, sourdough, and cow dung. Besides, they are also common inhabitants of human mouth, gastrointestinal and urogenital tracts, and stools (Gobbetti & Minervini, 2014). This therefore reflects that these bacteria are facultative anaerobic organisms and have a high tolerance for pH (acidity) and a wide range of temperatures. They have also been demonstrated to provide host health benefits when consumed due to many bioactive metabolites produced by them. Accordingly, many of these bacteria are considered to be probiotics (Hill et al., 2018). Their rod-shaped cells have a size range of 0.7-1.1 x 2.0-4.0 μm, often with square ends, which occur singly, in pairs, or in chains. The G + C content of their DNA is 45–47%. As an unique trait among the lactic acid bacteria, *L. casei* strains can utilise substrates, such as gluconate, malate and pentitols (Gobbetti & Minervini, 2014). *L. casei* is among the most studied lactic acid bacteria species in relation not only to their health potentials, but also their potential commercial and industrial applications. For instance, they are commonly used to greatly enhance the texture and flavour of foods like cheese. *L. casei*, *L. paracasei* and *L. rhamnosus* form a closely related group known as '*Lactobacillus casei* group', the species of which cannot be distinguished and discriminated from each another by 16S rRNA gene sequence analysis (Hill et al., 2018).

Lactobacillus plantarum

By having the largest genome known among the lactic acid bacteria, *L. plantarum* has been proposed to be very flexible, versatile, and strongly adaptive to highly heterogeneous environments compared to the other lactobacilli (Landete et al., 2010; Lorenzo et al., 2018; O'Sullivan et al., 2011). *L. plantarum* bacteria most typically represent the microbiota of fermented plant-based raw materials like silage, sauerkraut, pickled vegetables, brined olives, and sourdough. In addition, cow dung, dairy,

meat, fish, human and animal mucosa (e.g., oral, gastrointestinal, vagina), stools and sewage are also important habitats for these aerotolerant anaerobic bacteria. Their cells are typically straight rods with rounded ends, 0.9–1.2 x 3.0–8.0 μm, occurring singly, in pairs, or in short chains. The G + C content in their DNA has been reported to be 44-46 mol% (Corsetti & Gobbetti, 2002). *L. plantarum* strains hold promise as probiotic since daily intake of these bacteria has been associated with significant decrease in the number of pathogenic bacteria and infections in digestive tract of healthy volunteers. Moreover, they have also been shown to have a capability of reducing LDL-cholesterol and fibrinogen levels in blood, and a capability of producing beneficial metabolites, such as cis-9, trans-11-octadecadienoic acid and trans-9, trans-11-octadecadienoic acid in large amount. Also, there have been a finding that *L. plantarum* isolated from dairy sources tends to show a broad spectrum of antibiotic resistance, such as tetracycline, erythromycin, ampicillin, penicillin G, ofloxacin, vancomycin, norfloxacin, and ciprofloxacin.

It has been suggested many times that the use of *L. plantarum* as a probiotic could be preferable to that of other lactic acid bacteria due to the convenience in production, high-level genetic accessibility, and effective performance in the gastrointestinal tract (Corsetti & Valmorri, 2011).

2.2.2.2 Bifidobacterium

As is the case with *Lactobacillus*, *Bifidobacterium* (*B.*) strains also attract significant attention for their promising application as probiotic organisms (Plumbridge, 2009). With regard to phenotypic characteristics, they are also Gram-positive, saccharolytic, non-spore forming, non-motile, catalase-negative bacteria, and likewise, grow most preferably at temperature of 37 – 41 °C and at a pH of 6.5-7.0 (Hoover, 2014; Roy, 2019; Shah, 2011). In addition, they have many same habitats as lactobacilli, such as the human gastrointestinal tract and some fermented dairy products. However, there are many properties that distinguish them from the lactic acid bacteria. First of all, this genus is phylogenetically related to the phylum of *Actinobacteria*, with having a relatively high guanin + cytosine content (up to 67%) in their genome (Donohue & Gueimonde, 2011; Pyclik et al., 2020).

Although they can also produce lactic acid, another metabolite like acetic acid is also produced simultaneously in equal or even more amount (3:2) (Roy, 2019). For this, they particularly utilise fructose-6-phosphate phosphoketolase pathway rather than the homo- or heterofermentative ones typical for lactobacilli (Donohue & Gueimonde, 2011). As for their morphology, bifidobacteria normally appear as characteristic ‘bifid’, branched or Y/V-shaped rods. However, they can have a pleomorphic attribute under adverse growth conditions (Ferrario et al., 2019; Pyclik et al., 2020). With

a few exceptions (e.g., *B. animalis* subsp. *lactis*, *B. asteroides*), they generally require strictly anaerobic conditions for their optimal growth, because of which the industrial application of them is generally more challenging than that of the *Lactobacillus* ones (Ferrario et al., 2019; Roy, 2019). Further to this, bifidobacteria are generally demonstrated to be more vulnerable to strong acidic environment as well (do Carmo et al., 2018). Bifidobacterial strains – along with lactobacilli strains – are among the first colonising and dominant members of the human gut microbial community from the very first breast-feeding moment, thus play a vital role in the modulation of the mucosal physiology and innate immunity during new-born or neonatal period. Later on, their dominance normally decreases with ageing and with the commence of more solid and greater variety of diet, although some of these bacteria are still present in adulthood (Conlon & Bird, 2015; Ferrario et al., 2019; Gomes et al., 2014).

Bifidobacterium animalis* subsp. *lactis

B. animalis subsp. *lactis* – especially the strain Bb-12 – is currently the world's most documented and utilised probiotic species among bifidobacteria. It should be noted that *B. animalis* subsp. *lactis* itself is more commonly referred to as *B. lactis*. This species can greatly colonise the mammalian colon and are typically found in fermented dairy medium, animal and human (including infant) feces, and in sewage. Their DNA contain G+C in around 61 mol% (Jungersen et al., 2014; Mattarelli & Biavati, 2018). There have been several clinically supported findings showing that they can support human health through e.g., immune modulation, alleviation of antibiotic-derived side effects, improvement of bowel movement, inhibition of pathogens and enhancement of gastrointestinal microbiota and barrier function (Eskesen et al., 2015; Jungersen et al., 2014; Quigley, 2017). Furthermore, they have been demonstrated to have excellent gastric acid, bile, oxygen tolerance, to produce bile salt hydrolase and to adhere strongly to mucus, as compared to other *Bifidobacterium* species (Jungersen et al., 2014; Ruiz et al., 2012).

For being few of the most aerotolerant species of *Bifidobacterium*, along with having a good resistance to stressful conditions, *B. animalis* subsp. *lactis* is one of the most frequently used bifidobacteria in the functional food industry (Ruiz et al., 2012).

2.2.3 Therapeutic roles of probiotics in human body

Up to date, a wide range of human health effects have been proposed for probiotics (Figure 1). Although, it should be noted that the exact mechanism of action for most of these health claims has

not been sufficiently elucidated yet and more human clinical trials are needed to support them. However, it is known that probiotic effects often vary at a strain specific level – meaning that there is no probiotic strain that exerts all these benefits on human body – and can be induced by multiple level and factors. The most highlighted mechanisms of probiotics are re-establishment and maintenance of normal gut microbiome composition, which can confer numerous beneficial effects on host physiology, such as improving its gastrointestinal health (Cook et al., 2012; Khoder et al., 2016; Lv et al., 2021; Mazloom et al., 2019; Saarela, 2017; Sharifi-Rad et al., 2020; Syngai et al., 2016; Wang et al., 2016). To this end, probiotics can act in several ways: (1) promoting the growth of beneficial gut microorganisms by supplying them with nutrients; (2) suppressing pathogenic microorganisms by competing for essential nutrients and mucosal binding sites in the gut, and (3) by exhibiting antimicrobial factors (e.g., lowering pH of the gut environment, secreting organic acids, bacteriocins, bioactive peptides) to inhibit the *in situ* growth of these microorganisms (Sharifi-Rad et al., 2020; Sousa e Silva & Freitas, 2014; Syngai et al., 2016; Zoumpopoulou et al., 2018). In specific, the fall in pH derived from the secretion of organic acids like SCFA (short chain fatty acids) can re-establish the ideal ecological environment for the beneficial gut microbes and thereby induce the repopulation of those bacteria (Levy & Shah, 2011; Lockwood, 2008).

Enhanced defence of the normal gut microbiota, along with their established homeostasis, is utmost importance as several factors like antibiotic therapy, stressful and unhealthy lifestyle (e.g., smoking, lack of exercise), poor diet, and ageing can disturb these conditions (dysbiosis) (Conlon & Bird, 2015; Gomes et al., 2014; Sharifi-Rad et al., 2020; Zoumpopoulou et al., 2018); as a result, several gastrointestinal disorders like chronic inflammation and colorectal cancer can be induced (Śliżewska et al., 2020). In addition to the above-mentioned ones, probiotics have also been reported to exert many beneficial effects on extra gastrointestinal health. Majority of them are closely related to their modulation role on the normal gut microflora (Figure 1) (Sharifi-Rad et al., 2020).

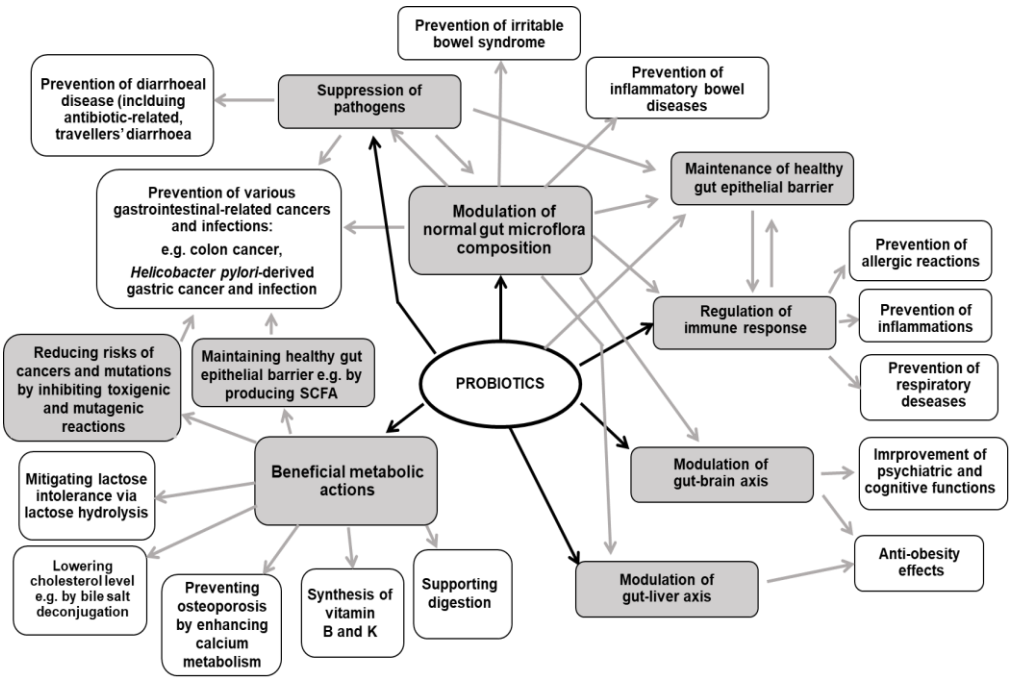


Figure 1. Proposed (not comprehensive) effects of probiotics on human health, along with mechanisms of action (grey) (Mazloom et al., 2019; Tamime & Thomas, 2017; Syngai et al., 2016; Wang et al., 2016; Lv et al., 2021; Khoder et al., 2016; Sharifi-Rad et al., 2020; Tripathi & Giri, 2014).

2.3 PREBIOTICS

Prebiotics are the group of nutrients that are non-digestible but can serve as selective substrates for the beneficial gut microbiota and probiotics, thereby conferring positive effects on the healthy balance of this specific microbiota and on human health. To date, most documented or proposed prebiotics are carbohydrates, including resistant starch, inulin, pectin, different oligosaccharides (e.g., galacto- and fructooligosaccharides), lactulose, lactosucrose, and many more (Chung et al., 2017; Davani-Davari et al., 2019; Martinez et al., 2015; Panesar & Bali, 2016; Tacer-Caba & Nilufer-Erdil, 2019). Potential prebiotic sources are generally fibre-rich foods, including oats (ß-glucan) and beetroot (pectin) (Henning et al., 2017; Rivera-Espinoza & Gallardo-Navarro, 2010; Sethi et al., 2016). The following sections outline the general properties of those prebiotics that I studied specifically in the present research work. In the case of **resistant starch,** it is discussed in Section 2.5.5.3.

2.3.1 Lactulose

Lactulose (ß-1,4-galactosylfructose) is a non-digestible, non-absorbable synthetic disaccharide that is widely used as a laxative to treat constipation, and also plays an important role in the treatment of portosystemic and hepatic encephalopathy. Synthetically, it is derived from lactose with the alkali isomerisation of the glucose moiety into fructose, generating the galactosyl β-(1→4) fructose form (Tungland, 2018). It has been shown that lactulose is usually converted to acetic, lactic and formic acids by the lower intestinal flora which contributes to the considerable pH drop in colon and stool (Levy & Shah, 2011; Lockwood, 2008).

2.3.2 Lactosucrose

Lactosucrose (β-4 ′galactosylsucrose) is a synthetic trisaccharide formed by either transfructosylation of lactose under the fructansucrase activity, or by transgalactosylation of sucrose under the β-galactosidase activity. Lactosucrose is used as a low-calorie sweetener with a relative sweetness of 0.3-0.6 compared to sucrose (Gänzle, 2011). Based on both animal and human trials, some studies have demonstrated its prebiotic potentials on the growth of bifidobacteria and also on the normal balance of beneficial gut microbes (Díez-Municio et al., 2014; Long et al., 2019; Mu et al., 2013). In fact, it is also reported that bifidobacteria – through their extracellular β-galactosidase and β-fructofuranosidase activity – can utilise and grow on lactosucrose better than oligosaccharides like fructooligosaccharides (Long et al., 2019). Lactobacilli can utilise it by their extracellular fructansucrase activity, and by the subsequent intracellular hydrolysis with ß-galactosidases (Gänzle, 2011). However, the prebiotic activity of lactosucrose has not been yet established as well as that of lactulose, galacto- or fructooligosaccharides, and more studies is still needed to sufficiently prove that (Silvério et al., 2015; Villamiel et al., 2014).

2.4 SIGNIFICANCE OF SUFFICIENT PROBIOTIC VIABILITY

As the most common definition (FAO/WHO, 2001) also indicates, it is utmost important for probiotics to be delivered to gastrointestinal tract (human gut microbiota) in highly viable form so that they can effectively exert their therapeutical effects on human body. To this end, it is often recommended that probiotics should be consumed at the daily dose of 10^8-10^9 CFU, along with food / supplement products containing a minimal viable cell concentration of 10^6-10^7 CFU/g or mL (Fredua-Agyeman et al., 2017; Kailasapathy, 2002; Yao et al., 2020). Further to this, Minelli and Benini (2008) additionally indicated the optimal presence of viable probiotics of 10^6-10^7 CFU/mL in the small

intestine and 10^8-10^9 CFU/g in the colon. However, satisfying this criterium has emerged to be challenging since the viability of probiotics can be threatened by a number of stress conditions during the manufacturing processes, transportation and storage of the carrier foods, and during the subsequent passage through the digestive tract. Owing to these viability loss tendencies, the actual number of live probiotics delivered to gastrointestinal tract, and thereby the claimed therapeutic effects of their carrier probiotic products can be questionable (Corona-Hernandez et al., 2013; Kailasapathy, 2002). Several studies have even shown that many commercial probiotic products – including foods and supplements – failed to maintain the sufficient viability of the cells under simulated gastrointestinal conditions (Dodoo et al., 2017; Fredua-Agyeman & Gaisford, 2015; Wills, 2012).

2.4.1.1 Adverse conditions during food processing

When probiotics are involved in the food manufacturing processes, they are often exposed to several physical harsh conditions, such as heat exposure during the processes like pasteurisation, sterilisation and hot air drying (e.g. spray drying); osmotic stresses by the effect of dehydration or thawing; mechanical stresses by the effect of freezing, excess mixing/agitation or spraying; and high level of oxygen or relative humidity exposure. Thermal processes like pasteurisation and sterilisation are frequently applied in the food industry to inactivate the spoilage and pathogenic microorganisms in the carrier food products. However, these heat treatments can simultaneously be deleterious to probiotics that are rather mesophilic or moderately thermophilic (Tripathi & Giri, 2014; Yao et al., 2020).

2.4.1.2 Adverse conditions during food storage

In the case of transportation and especially long-term (household) storage in carrier food products, the following environmental factors are expected to affect the stability of live probiotics: temperature; the level of relative humidity, water activity, oxygen, peroxide; pH; concentration of organic acids and other harmful ingredients present in the particular carrier food medium (Tripathi & Giri, 2014; Yao et al., 2020). In specific, most probiotics are sensitive to very low pH, along with the bactericidal effect of organic acids present in the food matrix or derived from the acidification activity of probiotics during the storage period. Based on the most accurate explanation, this acid sensitivity is possibly caused by the inhibition of glycolysis under acidic conditions, after which the lack of ATP synthesis can make their F_0F_1-ATPase system dysfunctional for the expulsion of H^+ accumulated in the cell (Corcoran et al., 2005; Cotter & Hill, 2003). Furthermore, the high degree of oxygen exposure can be detrimental to some oxygen-sensitive (anaerobic) probiotic strains as the absence of the electron transport chain in their metabolism can cause the toxic intracellular accumulation of oxygen

(Talwalkar & Kailasapathy, 2004). According to some studies, probiotics may show susceptibility to several additives like flavourings, aroma compounds and sweeteners that are widely used in many different food products (De Prisco & Mauriello, 2016; Vinderola et al., 2011). Regarding the high humidity/moisture, it has also been reported that storing under these specific conditions can trigger probiotics to early exert intensive physiological activity, which in turn inactivates them before being consumed (Yao et al., 2020). On this aspect, storage temperature also plays an important role in the probiotic survival, considering that the metabolic activity occurring in the carrier food is more likely decelerated under refrigerated storage conditions (Zhao et al., 2018).

With regard to storage in **fruit, vegetable or cereal-based food beverages**, the high sugar and other nutrient content can make these them appropriate for growing and maintaining probiotics therein (Aspri et al., 2020; De Prisco & Mauriello, 2016). However, there are several risk factors that may make the application of these products as probiotic carrier challenging, including either their natural antimicrobial activity – through e.g., peptide, phenolic and organic acid components – , low pH or dissolved oxygen-rich environment (Aspri et al., 2020; Chandrasekara, 2019; De Prisco & Mauriello, 2016; Gawkowski & Chikindas, 2013). Furthermore, these non-dairy food products are often stored and marketed at room temperature, which can pose additional challenge for probiotic stability (Vinderola et al., 2017).

2.4.1.3 Adverse conditions during digestion processes

After being ingested with carrier foods, probiotics confront a number of different stress factors during the passage through the upper gastrointestinal tract, especially in the stomach and in the small intestine. First, when they arrive to stomach, they must survive the strong acidic conditions (pH = 1-3) that characterises the gastric fluid; in addition, the enzymatic digestion (mainly of pepsin), the presence of high ionic strength and the intensive mechanical churning of stomach can further exacerbate the stress conditions during this digestion phase. Later in the small intestine, it is particularly the bile acids and the pancreatic digestive enzymes (e.g., trypsin, chymotrypsin, proteases, amylase, lipase) that can challenge the survival of probiotics (Charteris et al., 1998; Yao et al., 2020). In specific, bile acids have been shown to confer a potent antimicrobial activity mainly by dissolving cell membrane lipids and by impairing genetic materials in cells. Although some probiotic strains can have an ability to deconjugate the bile acids, the resultant free bile acids may henceforth acidify the cytoplasm in the cells (Begley et al., 2006; Hay & Zhu, 2016; Kurdi et al., 2006; Yao et al., 2020).

When designing delivery systems for probiotic encapsulation with the aim of improving their gastrointestinal tolerance and controlled release it is necessary to evaluate these microencapsulated

systems with *in vivo* or *in vitro* gastrointestinal assays. It is certain that *in vivo* methods would undoubtedly provide the most informative findings on the behaviour of the microencapsulated systems and would also speed up the process of bringing any of these products to market (Cook et al., 2012). However, there are several difficulties associated with the *in vivo* approaches, considering the limitations in experimental design, the difficulties in data interpretations, the high cost of equipment and labour, the wide inter-individual variations of the human gastrointestinal physiology, the lack of certified reference standards to compare data among relevant studies and, last but not least, the ethical concerns (Verhoeckx et al., 2015). In this sense, the initial evaluation of the microencapsulated formulations is typically conducted *in vitro*. Several *in vitro* gastrointestinal model systems have been developed to simulate the physiological conditions of the human gastrointestinal digestion, the simplest being the static methods. To date, the majority of the human gastrointestinal simulations applied in the previous life science-related studies are very simplified, in which the applied simulated gastric fluid is mostly a solution of HCl and salts, adjusted to pH = 1.0-2.0 (Cook et al., 2012). However, the comparison of the results obtained by different related *in vitro* studies can be sometimes difficult due to inconsistencies in the composition and pH of the simulated gastrointestinal fluids in these applied models. To address this specific problem, the COST Action Infogest network of researchers recently developed and published a static model that aimed to both elaborate and harmonise the previous *in vitro* protocols simulating human digestion (Minekus et al., 2014). For an even better validation of efficacy of the microencapsulated formulations *in vivo*, a dynamic gastrointestinal model system (e.g., TNO, SHIME, or SIMGI models) can be considered as an alternative methodology due to the feasibility of automatic, real-time control of different parameters (e.g., pH, flow of digestive contents, enzyme injection), which provides much more accurate mimicking of the human *in vivo* digestion than the static ones do (Verhoeckx et al., 2015).

So far, discovering new stress-resistant strains of probiotic bacteria – either naturally or by genetical modification – and applying microencapsulation technology have been suggested to be the most feasible approaches to address the above-mentioned problems (Călinoiu et al., 2019). Although, the use of genetically modified probiotic bacteria in food manufacturing setting and in human subjects may come with some health and technological risks, considering the possible dissemination of modified stains, plasmids and recombinant genes; the low public acceptance, and also considering that the ecology of human gut is not fully known and understood yet (Cummins & Ho, 2005; Plavec & Berlec, 2020).

2.5 MICROENCAPSULATION TECHNOLOGY

Microencapsulation or, in other words, design of micro delivery (capsule) systems has so far emerged as the most potential approach to protect the viability of probiotics from unfavourable environments and thereby enhance the therapeutic efficiency of probiotic products. Main principle of this technology is that bioactive agents like probiotics are surrounded by a polymer wall or embedded within a polymer matrix, and as such, they can potentially be protected through this barrier system. The material encapsulating the core material(s) is also often called as shell, coating, membrane, film, external phase, carrier, supporting or just encapsulating agents, while core material(s) is also called interchangeably as fill, payload, active phase, internal phase or encapsulated agents (Frakolaki et al., 2020; Shekhar et al., 2010; Yao et al., 2020). Technology can be considered as microencapsulation if core materials are encapsulated into tiny capsules in size range from 0.2 to 5000 μm. Although, in some studies, this size range has also been defined to be 1 - 1000 μm or 3 - 800 μm (Ahn et al., 2010; Considine & Considine, 1995; Pech-Canul et al., 2020; Sousa e Silva & Freitas, 2014). Accordingly, capsules with smaller size (< ~ 1 μm) are referred to as nanocapsules and the process itself is as nanoencapsulation. However, this latter technology is not suitable for probiotic encapsulation as their cell size is typically in the micron range (Capelezzo et al., 2018; Frakolaki et al., 2020; Whelehan & Marison, 2011).

2.5.1 Controlled gastrointestinal delivery of probiotics

Other than protective functions, applying capsule systems can potentially enable a controlled (targeted and sustained) release of the probiotic core content into the human gut, especially the large intestine, where they can proliferate and exert their health effects (Cook et al., 2012; Haghshenas et al., 2015). Depending on the applied encapsulating materials, the release mechanism in the gastrointestinal tract can manifest as either degradation, disintegration, swelling or dissolution of the capsule formulations, which can be triggered by some stimuli such as pH, time, enzyme activity, peristaltic pressures or microbial fermentation (Cook et al., 2012; Malekjani & Jafari, 2020). Another contributing factor for controlling gastrointestinal delivery and release of probiotics is to design capsule systems with specific mucoadhesive traits, assuring the sufficient residence and colonisation of probiotics on the gastrointestinal epithelial tissue.

Mucoadhesion is a trait or process with which a polymer (e.g., capsule wall/matrix material) can adhere to the mucosal membrane on the epithelial tissues via certain interaction with its mucins (Khutoryanskiy, 2011). Mucins as large, glycosylated proteins are secreted from the epithelial cell

surface to form a viscoelastic mucus gel layer thereon that can act as a protective barrier to the underlying epithelium against toxic challenges (e.g., chemical, enzymatic and pathogenic factors), among others. This glycoprotein can confer a negative charge nature to mucin through its sialic acid and sulphate residues fully ionised at pH > 2.6 (Varum & Basit, 2014). The exact mechanism behind the mucoadhesive interaction is not fully elucidated yet. However, many theories have been proposed so far (Cook et al., 2017; Khutoryanskiy, 2011; Komati et al., 2019). One of these theories is adsorption which involves the chemical bonds between the mucin and the polymer, such as Van der Waals, hydrogen, and hydrophobic forces. However, with an appropriate chemical characteristic of polymers, this interaction is also possible with a stronger covalent bond. Another proposed mechanism is the electronic theory which is based on the ionic interaction between the positively charged polymer network and the negatively charged mucus layer. Aside from them, diffusion, mechanical, wetting and dehydration-based mechanisms may also possibly play an important role on the mucoadhesion. In fact, some combinations of these presented theories can also be considered.

2.5.2 Applications of encapsulation technology in food industry

Microencapsulation technology along with its beneficial potentials have first been recognised by pharmaceutical industry which exploited it to develop different drug dosage forms with improved therapeutic functions and controlled delivery (Henrique Rodrigues do Amaral et al., 2019; Khandbahale, 2020; Lee et al., 2018; Maharaj et al., 2015; Mania, 2013; Singh et al., 2010). Although the encapsulation technology – including both micro and nanoencapsulation – is not yet well adapted for food commercial applications, it has also found many potential applications in this specific field, on which remarkable scientific progress has been achieved. For instance, a wide variety of (micro and nano) delivery systems have long been developed to encapsulate food chemical ingredients, such as antioxidants, vitamins, polyphenols, micronutrients, lipids, enzymes, and food additives (e.g., flavourings, colour agents), among others. In their cases, encapsulation has been demonstrated to improve either their bioavailability, water solubility, thermostability, storage stability, functionality, stability against gastrointestinal digestive enzymes, or controlled release. On another aspect, it can potentially be used to mask unpleasant sensory characteristics (e.g., taste, odour, colour) of foods and also to inhibit oxidative reactions therein (Chavarri et al., 2012; Kwak, 2014; Rahila Parveen & Preetha, 2020; Wen et al., 2014; Yang et al., 2020; Ye et al., 2018). Most recent studies have also focused on the development of effective micro delivery systems for probiotics (see examples in Table 2). However, compared to chemical compounds, it can be a bit more challenging when it comes to

selecting suitable materials and techniques for encapsulating such live organisms, since the viability issue is also an important consideration in this case.

2.5.3 Criteria for designing ideal probiotic delivery systems

As it is obvious, high probiotic-loading yield of the microcapsules is an important requirement to assure the sufficiently high administration of viable probiotics. Accordingly, microencapsulation process should ideally be carried out under mild conditions (e.g., at ambient temperature, neutral pH, low mechanical stress, low presence of oxygen) to avoid the potential viability loss of probiotics, which, however, leads to limited choice of suitable encapsulation procedure and material (Pech-Canul et al., 2020).

The proper selection of encapsulating method and material is also decisive in many other aspects of developing ideal delivery systems. First, the adaptation of the micro delivery systems for food industrial applications can be easier if their mass production comes with low cost, simplicity, reproducibility, scalability, and no health risk (i.e., no hazardous or toxic chemical applications). As for selecting proper encapsulating materials, the following technological and safety criteria should be particularly met (Cook et al., 2012; Pech-Canul et al., 2020; Wandrey et al., 2010):

(1) do not harm neither the encapsulated probiotics nor the human health
(2) biocompatible
(3) biodegradable
(4) inexpensive
(5) available in mass amount
(6) good quality
(7) naturally occurring
(8) soluble in common solvents like water
(9) easy to handle
(10) thermostable
(11) do not impair the sensory characteristics of the carrier food
(12) have been long used in food industry

Furthermore, the size of the capsule particles is also an important consideration when incorporating them into food products, since too large particles can negatively affect the sensory characteristics (e.g., texture, flavour) of such foods. In this regard, some papers recommend capsule formation with the maximum size of 80 - 100 μm (Costa et al., 2014; Frakolaki et al., 2020). Also, it has been suggested

that too large capsules can longer retained in the stomach due to the small opening diameter of the pyloric sphincter (~ 1 - 2 mm during fed state) which controls the rate of gastric emptying (O'Malley & Ziessman, 2014; Wallace, 2015; Yao et al., 2020). As such, it also implies another advantage of delivering probiotics through proper micron sized capsule systems as compared to those large (> ~ 1 cm) pill forms typical for many commercial supplements. Last but not least, microencapsulated probiotics should adequately withstand the adverse conditions encountered during food manufacturing, storage, and digestion processes, and should be released exclusively in gastrointestinal tract (Dodoo et al., 2017; Sousa e Silva & Freitas, 2014; Tamime & Thomas, 2017; Yao et al., 2020).

In recent years, there have been several research carried out on the microencapsulation of probiotics for enhancing their resistance to stress environmental factors. As a result, a wide range of promising delivery systems have been devised in laboratory scale and assessed so far in terms of their effectiveness. However, the problem is that there are still many shortcomings in these systems regarding the food industrial and commercial applications, such as their high production cost, too large capsule size, low scalability, insufficient cell protection ability, the lack of elaborate processing requirements, or that the ingredients or polymer wall/matrix used for the encapsulation is not suitable for food applications (Yao et al., 2020). Thus, additional research works should be devoted to find a solution in order to fill the scientific gaps regarding the microencapsulation of probiotics.

2.5.4 Techniques for microencapsulation of probiotics

Among the numerous techniques developed for microencapsulation purposes (Mishra, 2015a), there are a few of them that can be considered specifically for microencapsulating probiotics, with involving many different physical and chemical principles. These techniques include gel forming-based techniques like extrusion and emulsification (Krasaekoopt et al., 2004; Muthukumarasamy et al., 2006; Sheu & Marshall, 1993); drying-based techniques like spray drying (Lee et al., 2004; O'Riordan et al., 2001), fluid bed drying (Penhasi, 2015), freeze drying (Rajam et al., 2015); electrospraying (Coghetto et al., 2016; Zaeim et al., 2017); spray chilling (also spray cooling or congealing) (Arslan-Tontul & Erbas, 2017; Okuro et al., 2013), layer-by-layer self-assembly deposition (Diaspro et al., 2002; Priya et al., 2011), compression coating (Chan & Zhang, 2005) and coacervation (Zhao et al., 2018). The following sections discuss the important characteristics of all those techniques that I applied and studied in my research work.

2.5.4.1 Gel forming-based microencapsulation techniques

Entrapping into hydrogel-based polymer matrix is the most commonly studied approach for probiotic microencapsulation. This hydrogel matrix is classically formed by ionotropic cross-linking of

polymers – normally hydrocolloids or hydrogel – in the presence of proper oppositely charged, monovalent or divalent ionic solution. The successful formation of gels is greatly determined by factors such as the good gelling capacity of polymers. In this regard, it has been indicated that either long or linear polymers tend to have better gelling capability (Cook et al., 2012; Kwak, 2014).

Extrusion technique

The main feature of this specific technique is that a concentrated hydrogel polymer solution containing cells is forced (extruded) through a capillary (e.g., needle or nozzle) and then fall into an appropriate ionic cross-linking solution in the form of droplets (Figure 2). In this solution, polymer droplets are externally gelled, that is, the cross-linking agents diffuse inward the polymer matrix (Cook et al., 2012; Costa et al., 2014; Frakolaki et al., 2020; Mishra, 2015a; Solanki et al., 2013). Extrusion-based encapsulation has been applied in the food industry for several years with the first case achieved for a flavour component, resulting in its better stability (Mishra, 2015a).

The reasons for the widespread use of this method are that it is simple, cheap (at least on laboratory scale), and it requires mild encapsulation conditions (e.g., ambient temperature and no necessary use of deleterious solvents) which is useful in the field of probiotic microencapsulation. Thus, high encapsulation yield can be easily achieved with it. Furthermore, particles can be produced in a very narrow size distribution with this technique. However, the typical particle / capsule size that can be achieved here is relatively large, generally ranging from 1 to 5 mm (Cook et al., 2012; Liu et al., 2020; Solanki et al., 2013). Besides, the range of encapsulating materials suitable for gelling process is limited. Although this encapsulation approach itself is difficult to be scaled up due to its slow capsule formation, there have been promising concepts emerged in the improvement of extrusion procedure. In specific, the use of jet-based modifications has been shown to aid the large-scale operation of the extrusion and also the reduction of capsule size even to a few hundred-micron range. Examples of these jetting systems include coaxial flow jetting, aerodynamically assisted jetting, nozzle vibration atomisation, spinning disk atomisation, jet-cutter atomisation, centrifugal atomisation and microfluidic technology, and electrostatic atomisation (see in Section 2.5.4.2) (Chavarri et al., 2012; Costa et al., 2014; Whelehan & Marison, 2011). However, the adaptation of these auxiliary systems for the industrial production should incur some additional costs.

Emulsification technique

Another widespread approach for gel-based encapsulation of probiotics is the emulsification or emulsion method. In this procedure the formation of gel capsules is basically derived from the ionic

cross-linking (gelation) of the aqueous polymer solution that emulsified in a larger volume of oil (Figure 2). Accordingly, cell encapsulation is assured when they are included in this polymer phase and dispersed together in this water-in-oil emulsion system. Based on the ionic gelation mechanism, there are two possible approaches for performing the emulsification-type gel capsule formation. One of these approaches is when the dispersed polymer phase is gelled by external (inward) gelation, that is, ions diffuse into the dispersed phase while the ionic solution is gently poured into the emulsion. Less commonly applied gelling mechanism is internal gelation, which has been particularly developed for sodium alginate-based dispersed phase. In this case, calcium carbonate (or any other insoluble calcite micro-crystals) is included in the alginate solution, which disintegrates to calcium ions and carbonic acid when organic acid is added to the emulsion system. As a result of it, alginate-based gel network is formed internally (outwards) by the freed calcium ions (Cook et al., 2012; Poncelet, 2001; Reis et al., 2006).

As is the case with the extrusion one, this encapsulation approach is likewise easy to use, does not require any special equipment or tools, and usually provide a high loading yield with live bacteria due to its mild conditions. Besides, it is also easy to scale-up. Although it allows the formation of much smaller capsules as compared to the extrusion method, much wider capsule size and shape variations is more typical here due to the polydispersity in the emulsion system. The possible size of each capsule is reported to range from 0.2 to 5000 μm with emulsification method. Furthermore, the necessary use of large amount of oil and surfactant (emulsifier), and the washing process of the gel particles to remove the remaining oil on their surface usually come with a high cost in case of the large industrial applications (Costa et al., 2014; Frakolaki et al., 2020; Haffner et al., 2016; Solanki et al., 2013). Thus, this is the reason for that this encapsulation process is rather limited to laboratory uses.

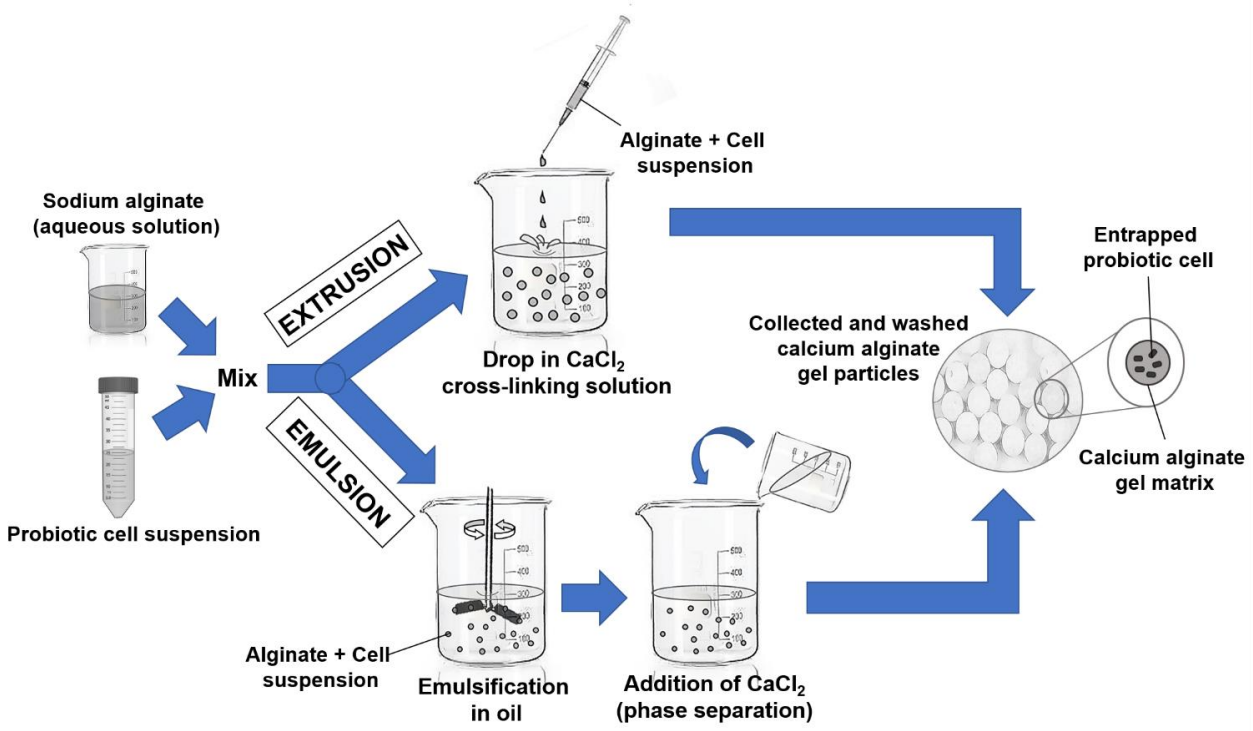


Figure 2. Schematic illustration of the extrusion and emulsion-based encapsulation of probiotic cells with alginate (as a representative encapsulating material)

2.5.4.2 Electrospray technique

Electrospraying, also known as an electrohydrodynamic atomisation, is an effective technology for mass fabrication of micro- or nanoparticles and which has found many applications in biological and medical industry, particularly in the production of controlled drug delivery systems, thin film deposits, superconductors, quantum dots, photo-ionic crystals, among others (Jafari-Nodoushan et al., 2015). The principle of this technology is that a high-voltage electric field is applied to a needle/nozzle tip through which a polymer (feed) solution is passed and becomes highly ionised by it; as a result, this charged polymer solution is forced to spray towards an oppositely charged grounded metallic collector, thereby forming micro- or nanoparticles thereon. At above a specific voltage level, the induced electrostatic tension between the tip and the collector pushes the meniscus surface of such liquid away from the tip, while overcoming its apparent surface tension; thus, this liquid is ejected out of the capillary in a stable Taylor cone-Jet form which broken up into much finer droplets (Bhushani et al., 2017; Jafari-Nodoushan et al., 2015; Wang et al., 2019). The mechanism of the micro-/nanoparticle formation by electrospraying technique is also illustrated in Figure 3. The flow of polymer solution through the capillary is usually controlled with the use of an air compressor or a

syringe pump. As the collector, it can be a metallic surface or a liquid bath in metallic dish. The latter case is typically used in those specific electrospraying processes where gel particles are produced, by including some hydrogel as the feed solution and cross-linking medium as the collector bath (Zaeim et al., 2017). Accordingly, this electrospraying can be considered as electro-jetting version of the above-mentioned extrusion method (see Section 2.5.4.1).

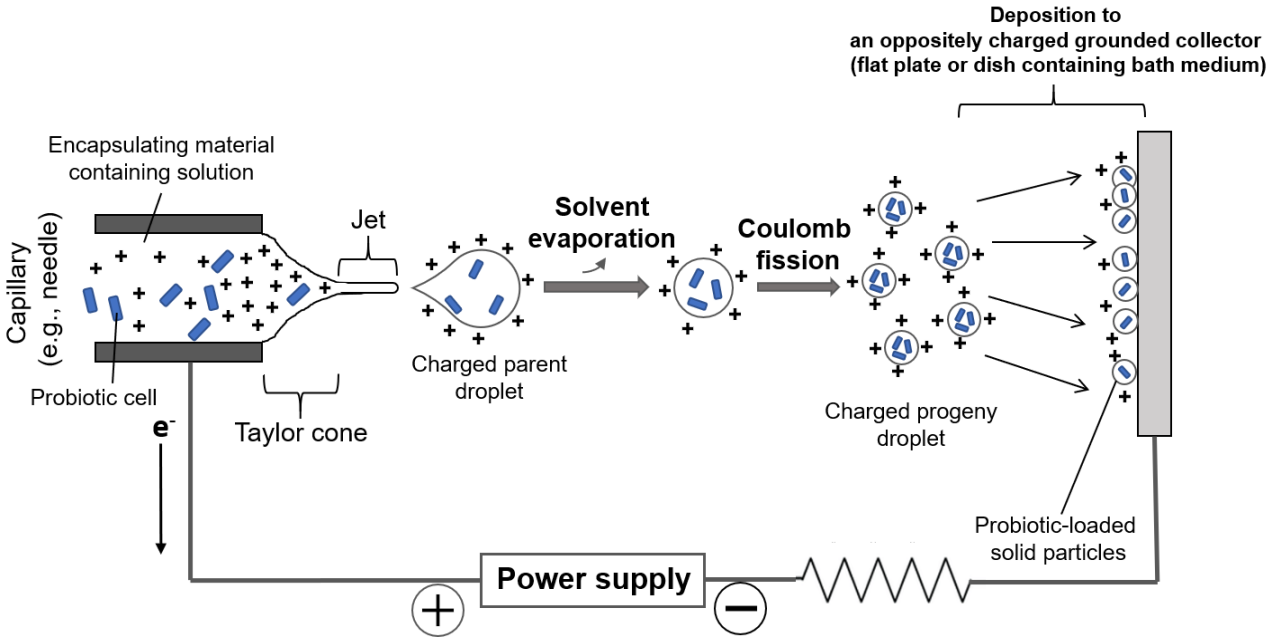


Figure 3. Mechanism of the electrospray-based formation of probiotic-loaded solid particles (adapted from the illustration of Banerjee & Mazumdar (2012))

As is the case with encapsulation of drugs, electrospraying has also attracted particular attention for encapsulating probiotic cells with offering the following numerous advantages: continuous one-step operation; ambient conditions in terms of temperature, pressure, chemicals; high particle production rate; potential adaptation to industrial scale; and the possibility of cell loading into micron sized particles (microcapsules). Further, it features many adjustable parameters like voltage level, flow rate of polymer solution, distance between the needle tip and the collector, needle gauge, concentration and conductivity of the feed solution, with which the characteristics and the size distribution of the end products can be tailored (Bhushani et al., 2017; Jafari-Nodoushan et al., 2015; Wang et al., 2019; Zaeim et al., 2018). With a coaxial configuration, there is even an opportunity to apply two liquids at once to form multilayer (core-shell) particles. The drawback of electrospraying approach is that only limited range of polymers are suitable for electrospraying, which is generally dependent on the

physical characteristics of the polymer, such as electrical conductivity, viscosity, surface tension and dielectric constant (Zaeim et al., 2018).

2.5.4.3 Coating technologies

Coating technologies is also another potential microencapsulation approach to protect the viability of probiotics and to assure their controlled release. In this case, some polymer coating is directly applied on the individual cells rather than entrapping multiple cells within the same polymer matrix or shell – which is more typical with the previously mentioned techniques. Polymer coating is also commonly applied on the ready-made capsules for possibly enhancing their protection ability. Besides, the application of the coating could also play a particular role in providing or improving mucoadhesive properties for capsules or probiotics.

Layer-by-layer self-assembly technique

Layer-by-layer (LbL) technique is a special approach for the coating-based microencapsulation, which based on the multiple and alternating electrostatic deposition of oppositely charged materials (e.g. polyelectrolytes) on the core material (e.g., bioactive compounds, cells) (Figure 4) (Diaspro et al., 2002; Priya et al., 2011). Although less typical, deposition itself can also be mediated through other mechanisms like covalent bonds, hydrogen bonds, acid-base, or coordination interactions (Guzmán et al., 2017). As an important benefit, this technique can offer many tailoring opportunities in the formation, which allows highly varied and precise design of LbL-assemblies in terms of their structural (e.g., thickness and permeability of the assembled layers), physicochemical properties, therapeutic functionality and thus, the controlled delivery. In this regard, many formation-related variables that can determine the resultant LbL-assembly system include the following: the type, nature and charge density of the building (e.g. polyelectrolyte) and the core materials, the quality of the solvent for the building and the core (template) materials, the pH and temperature used for the deposition process, among others. Additional main advantages and characteristics of this technique are its simplicity, robustness and reproductivity (Guzmán et al., 2017). LbL-assembly method has also found potential application for microencapsulating living cells, which was first applied and assessed by Diaspro and co-workers (2002) with yeasts.

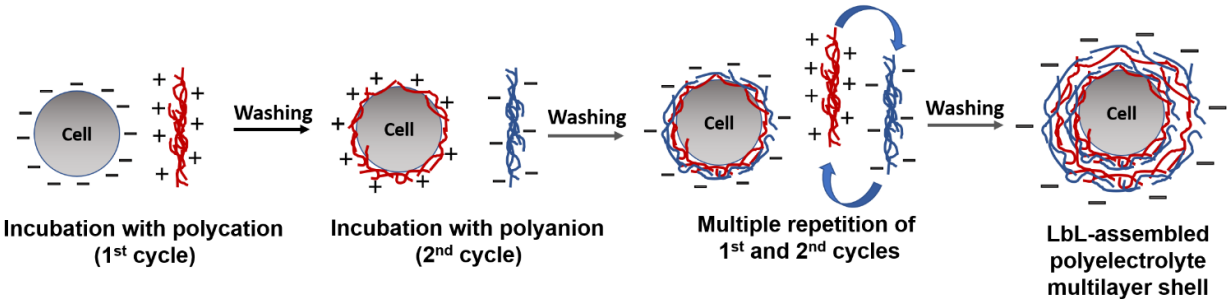


Figure 4. Schematic presentation of the electrostatic layer-by-layer (LbL) self-assembly process for coating probiotic cell with a polycation/polyanion multilayer shell

2.5.5 Materials for microencapsulation of probiotics

As more and more potential encapsulation techniques emerged for microencapsulation of probiotics, a huge variety of materials has likewise been proposed for entrapping, coating, or encapsulating probiotics. These include especially naturally occurring and bio-based materials, such as carbohydrates, proteins, lipids, etc. (Mishra, 2015b). Table 2 includes a list of many materials that successfully applied for the microencapsulation of probiotics.

Table 2. Examples of materials used for probiotic microencapsulation by each encapsulation technique

Method	Capsule material	Probiotic strains	References
Extrusion	Alginate	*L. acidophilus, L. reuteri, L. casei, L. acidophilus, B. bifidum, B. longum, B. breve,*	Afzaal et al., 2019; Chaikham & Apichartsrangkoon, 2012; Chandramouli et al., 2004; Cook et al., 2011; Gul & Dervisoglu, 2017; Iyer & Kailasapathy, 2005; Krasaekoopt et al., 2004; Lee & Heo, 2000; Li et al., 2011; Lotfipour et al., 2012; Muthukumarasamy et al., 2006
	Alginate-Pectin	*L. casei*	Sandoval-Castilla et al., 2010
	Gellan gum - Xanthan gum	*B. lactis, L. reuteri, B. bifidum, B. infantis, B. breve, B. adolescentis, B. longum*	McMaster et al., 2005; Muthukumarasamy et al., 2006; Sun & Griffiths, 2000
	Xanthan gum - Chitosan	*Pediococcus acidilactici*	Argin, 2007
	κ-carrageenan - Locust bean gum	*L. reuteri, B. infantis*	Muthukumarasamy et al., 2006; Ouellette et al., 1994
	Alginate - Starch	*L. acidophilus, L. reuteri, B. pseudocatenulatum*	Iyer & Kailasapathy, 2005; Muthukumarasamy et al., 2006; Teoh et al., 2011
	Alginate - Dextrin	*L. plantarum*	Mahmoud et al., 2020
	Alginate - Oligosaccharides (galacto -, isomalto, fructo-, xylo-oligosaccharides)	*L. fermentum, L. rhamnosus L. acidophilus, L. casei*	Krasaekoopt & Watcharapoka, 2014; Liao et al., 2019; Siang et al., 2019

Method	Capsule material	Probiotic strains	References
Extrusion	Alginate - Inulin	*L. acidophilus, L. casei*	Krasaekoopt & Watcharapoka, 2014
	Alginate – Plant extract (beetroot extract, ginger extract)	*L. plantarum, B. animalis*	El-Abd et al., 2018
	Alginate - Skim milk	*L. plantarum*	Mahmoud et al., 2020
	κ-carrageenan	*L. reuteri*	Afzaal et al., 2019
	Alginate - Whey protein	*L. plantarum*	Mahmoud et al., 2020
	Pullulan gum	*L. rhamnosus*	Jiménez-Pranteda et al., 2012
Emulsification	Alginate	*B. longum subsp. infantis, B. longum subsp. longum, B. animalis subsp. lactis, L. reuteri, L. casei*	Chen et al., 2012; Gul & Dervisoglu, 2017; Holkem et al., 2017; Ji et al., 2019; Muthukumarasamy et al., 2006; Sheu & Marshall, 1993; Yeung et al., 2016
	Alginate – Starch	*L. reuteri, L. acidophilus, L. plantarum, B. lactis*	Kailasapathy, 2006; Muthukumarasamy et al., 2006; Shafiei, 2018; Sultana et al., 2000; Xing et al., 2014
	Alginate - Whey protein isolate	*L. bulgaricus*	Chen et al., 2017
	Alginate - Skim milk	*L. acidophilus*	My Dong et al., 2020
	κ-carrageenan	*B. bifidum, L. lactis* ssp. *lactis*	Dinakar & Mistry, 1994; Sodini et al., 1997
	Gellan gum - Xanthan gum	*L. reuteri*	Muthukumarasamy et al., 2006
	κ-carrageenan - Locust bean gum	*L. reuteri*	Muthukumarasamy et al., 2006
	Cellulose acetate phthalate	*B. pseudolongum*	Rao et al., 1989
	κ-carrageenan - Skim milk	*L. plantarum*	My Dong, 2020
	Whey protein and milk fat	*B. breve*	Picot & Lacroix, 2004
	Whey protein	*B. bifidum*	Zou et al., 2012
	Casein	*L. casei*	Li et al., 2020
	Gelatin	*B. lactis*	Annan et al., 2008
	Maltodextrin - Gelatin	*Lactobacillus sp.* 21C2- 10	Sengsaengthong & Oonsivilai, 2019
Spray drying	Alginate	*L. bulgaricus*	Lee et al., 2004
	Skim milk	*L. paracasei, 31 Bifidobacterium* strains	Gardiner et al., 2002; Simpson et al., 2005
	Starch (readily gelatinised)	*Bifdobacterium* PL1	O'Riordan et al., 2001
	Cyclodextrin, Maltodextrin, Isomaltooligosaccharide	*L. brevis, L. acidophilus, B. adolescentis, B. infantis*	Lin et al., 2019
	Mik-based medium, Gum acacia	*L. paracasei*	Desmond et al., 2002
	When protein	*B. bifidum*	Zou et al., 2012
	Soy protein isolate - Alginate	*L. casei*	Hadzieva et al., 2017
	When protein – Maltodextrin/inulin	*L. rhamnosus*	Ying et al., 2012
	Sweet whey	*Bifidobacterium*	Pinto et al., 2017
	Gelatin	*L. rhamnosus*	Ying et al., 2012

	Capsule material	Probiotic strains	References
Spray chilling	Vegetable fat	*L. acidophilus, B. animalis subsp. lactis*	Bampi et al., 2016
	Fully hydrogenated palm and palm-kernel oil	*L. acidophilus*	Okuro et al., 2013
Electrospraying	Alginate	*L. acidophilus*	Laelorspoen et al., 2014
	Alginate - Chitosan	*L. plantarum*	Zaeim et al., 2017
	Alginate -Silica	*L. rhamnosus*	Haffner et al., 2017
	Alginate - Pectin	*L. plantarum*	Coghetto et al., 2016
	Acacia gum	*L. plantarum*	Zaeim et al., 2018
	Whey protein isolate - gelatin	*L. plantarum*	Gómez-Mascaraque et al., 2017
Layer-by-layer self-assembly	Carboxymethyl cellulose / Chitosan (alternatively)	*L. acidophilus*	Priya et al., 2011
	Chitosan / Alginate (alternatively)	*Bacillus coagulans*	Anselmo et al., 2016
Polymer coating	Alginate capsules coated with chitosan	*L. plantarum, L. acidophilus, B. longum subsp. infantis, B. longum subsp. longum, B. breve B. animalis subsp. lactis*	Chávarri et al., 2010; Cook et al., 2011; El-Abd et al., 2018; Iyer & Kailasapathy, 2005; Ji et al., 2019; Krasaekoopt et al., 2004; Liserre et al., 2007; Mahmoud et al., 2020; Nualkaekul et al., 2012; Sallehudin et al., 2017; Yeung et al., 2016
	Alginate – Starch capsules coated with chitosan	*L. acidophilus, L. plantarum, B. animalis subsp. lactis*	Iyer & Kailasapathy, 2005; Zaeim et al., 2019
	Alginate capsules coated with chitosan and carboxymethyl chitosan	*L. casei*	Li et al., 2011
	Alginate capsules coated with alginate	*L. casei, L. acidophilus, B. bifidum*	Iyer & Kailasapathy, 2005; Krasaekoopt et al., 2004
	Alginate capsules coated with poly-L-lysine	*L. rhamnosus, L. casei, L. acidophilus, B. bifidum*	Iyer & Kailasapathy, 2005; Krasaekoopt et al., 2004; Siang et al., 2019
	Alginate capsules coated with whey protein	*L. plantarum*	Gbassi et al., 2009
	Alginate capsules coated with zein	*L. acidophilus*	Laelorspoen et al., 2014

Based on the literature to date, **food hydrocolloids or gums**, especially the **polysaccharide**-based ones are the most widespread materials for encapsulation of probiotics due to their common usage in food industry, safe biodegradation residues, good solubility in water, and their diverse molecular and functional characteristics from various monomer compositions and substituent groups. Besides, there is also an opportunity to easily modify the substituent groups to form artificial or semi-natural polysaccharides with a specific custom-tailored characteristic. These applied hydrocolloids/gums can be sourced from nature or by chemically modifying native polysaccharides (Liu et al., 2020; Mishra, 2015b; Pech-Canul et al., 2020). According to their charge nature, polysaccharides can be classified as anionic, cationic, non-ionic, and amphoteric. This specific characteristic is an important

consideration for such encapsulation mechanisms as ionotropic gelation or electrostatic-based deposition. Anionic types are typically those polysaccharides that carry negative charges (carboxyl groups) at pH above their pK_a, while cationic ones are those that carry positive charges (amino groups) at pH below their pK_a. Amphoteric ones can exhibit both charge characteristics at the same time (Pech-Canul et al., 2020).

Besides polysaccharides, another widely studied encapsulating materials are **proteins**, including the animal (e.g., gelatin, whey protein, casein) and the vegetable originated ones (e.g. soy proteins) (Annan et al., 2008; Gómez-Mascaraque et al., 2017; Reid et al., 2007; Ying et al., 2012). Proteins are very large and complex molecules that as encapsulating materials can offer biocompatibility, biodegradability, good water solubility, emulsifying and good gelling properties (Blanco & Blanco, 2017; Mishra, 2015b). Furthermore, they have been demonstrated to act as an effective coating barrier against external O_2 and CO_2. Also, they can exhibit a wide variety of chemical interactions with other encapsulating materials both intra- and intermolecularly, due to their diverse amino acid sequences. However, there have been only few cases reported for the sole application of proteins as encapsulating material (Pech-Canul et al., 2020), which is likely related to their high degradability rate when confront with the digestive activity of pepsin in the stomach. In fact, the inclusion and consumption of especially animal derived proteins can be deterring for those who are allergic to it (Mishra, 2015b). **Lipid**-based matrix has also been used for a few times, typically for spray chilling-based probiotic microencapsulation (Arslan-Tontul & Erbas, 2017; Bampi et al., 2016; Okuro et al., 2013).

Without aiming to deal with an exhaustive list of potential encapsulating materials, the following sections cover only those ones which were specifically studied in this research work.

2.5.5.1 Anionic polysaccharides

Alginate has been the most extensively used materials so far for formation of probiotic-loaded microcapsules especially by ionic gelation and spray drying (El-Abd et al., 2018; Pech-Canul et al., 2020). Alginate is a polysaccharide that naturally found in the cell wall of brown algae species (*Laminaria* spp.) and mainly consists of linear copolymers of β-(1–4) linked D-mannuronic acid and α-(1–4) linked L-guluronic acid units (Pech-Canul et al., 2020). By carrying carboxyl groups on both monomers, alginate acts as an anionic polysaccharide at pH above its pKa (3.3-3.5), which tends to form an intermolecular cross-linked structure in the presence of divalent cations like calcium, cadmium, or zinc. In this case, a so-called ‘egg box’ shape (Figure 5) is formed through cross-linking the guluronic acid units of the alginate chain (Bruchet & Melman, 2015; Cook et al., 2012).

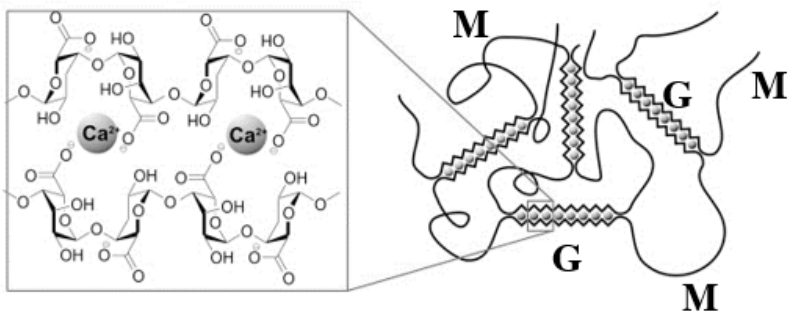


Figure 5. Schematic illustration of the 'egg-box' style binding of alginate mediated by calcium ions, and its chain association (Bruchet and Melman, 2015). M: mannuronic acid sequence, G: guluronic acid sequence

This polysaccharide has been reported to be effective for probiotic encapsulation thanks to its mild gelling conditions, simplicity, non-toxic, GRAS, relatively cheap, immunogenic, water solubility with forming high viscous solution, stabilising properties, biocompatible, and biodegradable by the human intestinal microbial community (Chen et al., 2005; Cook et al., 2012; Shilpi & Jain, 2016). According to some reports, alginate with an acid-gel character can stabilise the gel structure of microcapsules at pH below its pK_a (3.3-3.5) and thereby could be ideal for the gastric protection and the enteric delivery of probiotics (Cook et al., 2012; Draget et al., 1994). However, limitations have also been highlighted for calcium alginate gel matrix, including its high porosity which would increase the susceptibility of the entrapped cells to external adverse environments (Chen & Chen, 2007; Gombotz & Wee, 2012; Martín et al., 2015; Smidsrød & Skjåk-Bræk, 1990). According to several studies, co-encapsulation with or coating with other biopolymer, especially prebiotics, can potentially improve the cell protection ability of alginate gel matrix (Ashwar et al., 2018; El-Abd et al., 2018; Krasaekoopt & Watcharapoka, 2014; Krasaekoopt et al., 2003; Sabikhi et al., 2011; Samedi & Charles, 2019; Sultana et al., 2000).

Although less commonly studied than alginate, other hydrocolloidal polysaccharides have also been shown promising for entrapping and protecting probiotics against harsh acid and bile conditions in the gastrointestinal tract (Cook et al., 2012). These include, among others, pectin, carrageenan, gellan gum, xanthan gum and carboxymethyl cellulose, which are considered similar to alginate regarding their gelation mechanism, anionic charge, water solubility, biocompatibility and that they are also already in use for food applications. Although, especially carrageenan, gellan gum and xanthan gum have much higher molecular weight than alginate (National Center for Biotechnology Information,

2020), and their gelation can be also induced by temperature change besides the ionic approach (Chavarri et al., 2012).

Carrageenan is a high molecular-weight, linear polysaccharide consists of repeating disaccharide segments of D-galactose and 3,6-anhydro-D-galactose and mainly extracted from several species of red seaweeds by hot alkaline approach. As a thickening, gelling, consistency enhancing or stabilising agent, it is a widespread component in some food, pharmaceutical and cosmetic (e.g., toothpaste, hair, and skin care) products. According to the number and the position of sulphate groups on the galactose units, there are three commercial classes known, including κ- (monosulphated), ι- (bisulphated) and λ- (trisulphated) carrageenan (Chakraborty, 2017; Chavarri et al., 2012). Among of them, only the κ and ι forms allow gel forming thanks to their anhydrous bridges that can be used for divalent cationic cross-linking (e.g., Ca^{2+}, K^{+}). While ι-carrageenan generates soft and elastic gels in the presence of calcium ions, κ-carrageenan can form hard and firm gels in the presence of potassium ions. This may be the reason for that the latter one has been explored more for the probiotic encapsulation processes. Carrageenan generally dissolve in hot water and then solidify on cooling to < ~ 40-45°C (Chakraborty, 2017; Kulkarni & Shaw, 2016; Pech-Canul et al., 2020).

Further anionic polysaccharides that commonly used for encapsulating probiotics are bacterial-derived **gellan gum** and **xanthan gum**. The former one is produced by *Sphingomonas elodea*, while the latter one is produced by *Xanthomonas campestris*. **Gellan gum** is a poly-tetrasaccharide made up of two ß-D-glucose, one ß-D-glucuronate and one α-L-rhamnose. Gellan gum forms thermo-reversible hydrogels upon cooling, which mechanism is dependent on the composition of acyl groups of its glucose residues. While the highly acylated gellan gum (Gelrite) forms a soft, flexible hydrogels after cooling from 65°C even without the use of gelling cations, the low acylated gellan gum (Kelcogel) picks up a rigid hydrogel after cooling from 40°C (Pech-Canul et al., 2020). Gellan gum as additive is commonly applied in low-calorie version of some food products (e.g., fruit preparations, jams) (Jindal & Singh Khattar, 2018). The pK_a of low acylated gellan gum is reported to be 3.5, above which it shows anionic characteristics (Fasolin et al., 2013). On the other hand, **xanthan gum** is primarily composed of repeating penta-saccharide sequences formed by two mannose, one glucuronic and two ß-(1-4) linked D-glucose units (Cook et al., 2012; Pech-Canul et al., 2020; Sharma et al., 2014). The complexation of xanthan gum with divalent cations (e.g., Ca^{2+}, Mg^{2+}) takes place through its acetyl and pyruvate groups, among of which the high level of pyruvate group can specifically promote the gelling ability of xanthan gum. The pK_a value of these groups is typically around 4.5, conferring anionic characteristic to xanthan gum at pH above this value (Pech-Canul et al., 2020).

Combining different gums in some variations is a recent popular approach for enhancing the formation of gel matrix and for potentially improving its protection barrier (Table 2). For example, carrageenan – locust bean gum has been shown to give stronger ionotropic gel matrix and high protection against acidic conditions (Chakraborty, 2017; Riaz & Masud, 2013). Also, combining carrageenan with locust bean gum has been suggested to increase the tolerance of lactic acid bacteria to cross-linker KCl (during the ionotropic gelation process) (Krasaekoopt, 2013). Gellan gum on its own is not suitable for encapsulating mesophilic probiotics as it requires high gel setting temperature (> ~ 50°C) at high concentration (Camelin et al., 1993; Riaz & Masud, 2013). However, blending it with xanthan gum has been reported to lower the required setting temperature and improve the rheological quality of gellan gum gels (Sun & Griffiths, 2000).

As opposed to the previous polysaccharides, **carboxymethyl cellulose** is a semi-synthetic anionic polysaccharide formed by partially replacing the hydroxyl groups on the cellulose backbone (ß-D-glucose sequence linked with β-(1→4)-glycosidic bonds) with carboxymethyl groups during the alkali and chloroacetic treatment of cellulose (Pech-Canul et al., 2020). As such, this cellulose derivative can interact with cationic ions, thereby forming ionotropic gels through its acquired carboxylic groups (Patil et al., 2015) and also can better dissolve in water than cellulose. Cellulose itself is the most abundant polysaccharide in nature (Pech-Canul et al., 2020). In carboxymethyl cellulose form, it is also approved as GRAS and is commonly used as a thickener in several foods (e.g., cheese, salad dressings) (Holtzapple, 2003).

2.5.5.2 Cationic polysaccharide

Chitosan is another widely used encapsulating material in oral delivery applications for its minimally toxic, biocompatible and biodegradable properties (Anal & Singh, 2007). Chitosan is the only cationic (pH < 6.5) polysaccharide that can be found naturally. However, its semi-synthetic form is rather applied for industrial and commercial applications as its amount available in nature is very limited (Pech-Canul et al., 2020). The chemical composition of this polysaccharide consists of a linear backbone of randomly arranged β-(1–4)-linked D-glucosamine and N-acetyl-D-glucosamine residues, with amine functional groups (Cook et al., 2012; Nurunnabi et al., 2017). Artificially, chitosan is derived from partial N-deacetylation of chitin occurring in the exoskeletons of crustaceans and several bacterial parasites (Cook et al., 2012; Pech-Canul et al., 2020). Chitosan is typically soluble under acidic to neutral (pH < 6.5) conditions, and the viscosity and charge density of the resultant chitosan solution is also dependent on the molecular weight and the deacetylation degree of chitosan (Chavarri et al., 2012; Nurunnabi et al., 2017). Chitosan as cation can be gelled by ionotropic cross-linking with

anions such as tripolyphosphate (Giraldo et al., 2019), but also by chemical cross-linking with glutaraldehyde (Kildeeva et al., 2009) or by precipitating in NaOH solution (Sugashini & Gopalakrishnan, 2012). However, for the encapsulating probiotics, chitosan is more commonly applied as external coating layer on the ready-made capsules – made with anionic polymers like alginate – due to its possible broad-spectrum antibacterial effect and good film-forming ability (Chavarri et al., 2012). Coating alginate with chitosan has been explored in some studies to increase the survival of probiotics within alginate matrix under simulated gastric conditions (Chávarri et al., 2010; Cook et al., 2012; Nualkaekul et al., 2012). As a further merit, chitosan has been reported to exhibit excellent mucoadhesive characteristics due to its interaction with mucin via electrostatic attraction, hydrogen bonding and hydrophobic effects. For this reason, it has been widely used for mucoadhesive drug delivery applications (Khutoryanskiy, 2014; M. Ways et al., 2018).

2.5.5.3 Non-ionic polysaccharides

Among the natural non-ionic polysaccharides, locust bean gum, guar gum, starch, and its derivatives like maltodextrin and cyclodextrin have been applied for the encapsulation of probiotics. **Locust bean gum** – sourced from the seed of locust bean tree (carob tree) – is primarily made up by a long chain of galactose and mannose monomers (Mishra, 2015b). It is best soluble at high temperature of 60-90°C. In food industry, it is usually applied for improving its water binding property, smoothness, body and chewiness of frozen desserts, for speeding the curd formation of cheese, and for binding the moisture content of sausages and other cold meat products (Tomasik, 2003).

Starch is the most abundant and cheapest commercially available carbohydrate (Taniguchi & Honnda, 2009). With regard to chemical structure, it is made up by two different polysaccharides: linear (helical) amylose chain consisting of α-(1-4) linked D-glucose units and highly branched amylopectin consisting of main amylose chain coupled with several α-(1-6) linked D-glucose side chains (Pech-Canul et al., 2020). Starch is a main carbohydrate found in many plant-based foods like corn, potato, cereals, and many others. The ratio of amylose and amylopectin in starch, which can vary with different plant sources, plays an important role in determining the intrinsic characteristics of starch. For example, it has been suggested that starch with higher amylose content typically shows better resistance to enzymatic gastrointestinal digestion, which has been explained by the compact linear structure of amylose and the high presence of the hydrogen bonds that connect the glucose monomers. Thus, this sort of starch, namely **resistant starch** tends to reach the colon in intact form where it can potentially exert its prebiotic effect (Sarao & Arora, 2017; Zaman & Sarbini, 2015). Thanks to all these properties, resistant starch is demonstrated to potentially provide a good enteric

delivery and a better controlled (targeted) release of probiotics into the colon or anywhere within the intestinal tract (Chavarri et al., 2012). Besides, it is considered as one form of dietary fibre (Zhu, 2014), which consumption has been associated with improved gut mucosal barrier, prevention of inflammatory bowel disease and colon cancer, alleviation of constipation, prevention of diverticulosis and hemorrhoids, decreased cholesterol absorption, and increased LDL receptor expression in liver (Tomasik & Horton, 2012).

3 MATERIALS AND METHODS

3.1 APPLIED PROBIOTIC MICROORGANISMS

In my study, the strains of *Lactobacillus casei* 01, *Lactobacillus plantarum* NCDO 1752 and *Bifidobacterium lactis* Bb-12 were applied as model probiotic bacteria for the encapsulation studies. The bacteria were obtained from Chr. Hansen in freeze-dried DVS (commercial Direct Vat Set) form, except the *Lactobacillus plantarum* strain, which was among the culture collection of UK National Collection of Dairy Organisms (Reading).

3.2 APPLIED MATERIALS AND SOLUTIONS

3.2.1 Applied bacterial growth media

L. casei 01 and *L. plantarum* NCDO 1752 bacteria were grown in MRS (de Man Rogosa Sharpe) medium. For propagating *B. lactis* Bb12, TPY (Trypticase Phytone Yeast extract) medium was applied. These growth media were prepared as the following recipes show in Table 3 (De Man et al., 1960; Scardovi, 1981).

Table 3. Composition of MRS broth used for growing *Lactobacillus* strains and TPY broth used for growing the *Bifidobacterium* strain

MRS		TPY	
Component	**Amount**	**Component**	**Amount**
Proteose peptone	10 g	Trypticase peptone (BBL)	10 g
Beef extract	8 g	Phytone peptone (BBL)	5 g
Yeast extract	4 g	Glucose	5 g
Glucose	20 g	Yeast extract	2.5 g
Sodium acetate	5 g	K_2HPO_4	2 g
Triammonium citrate	2 g	$MgCl_2 \cdot 6\ H_2O$	0.5 g
Magnesium sulfate	0.2 g	Cystein HCl	0.5 g
K_2HPO_4	2 g	$ZnSO_4 \cdot 7\ H_2O$	0.25 g
Tween 80	1 mL	$CaCl_2$	0.15 g
Manganese sulfate	0.05 g	$FeCl_3 \cdot 6\ H_2O$	0.03 g
Distilled / Deionised water (DW)	1000 mL	Tween 80	1 mL
		Distilled / Deionised water (DW)	1000 mL

The final pH value of these broths was pH 6.8-7.0.

For solidifying these growth media to agar, 15 g/L of bacteriological agar (Sigma Aldrich) was added to the above liquid solutions. Before use, all these growth media – either with or without agar – were sterilised by autoclaving (at 121°C for 15 min).

3.2.2 Saline solution

0.85 % (w/v) saline solution, used as a diluent in serial dilutions, was prepared by dissolving 0.85 g NaCl in 100 mL distilled/deionised water (DW). This solution was then dispensed into separate test tubes in 4.5-4.5 mL volumes and brough to sterilise in autoclave (at 121°C for 15 min).

3.2.3 Peptone water and PBS

0.1 % (w/v) peptone water, used for suspending and storing encapsulated/unencapsulated probiotic bacteria, was prepared by dissolving 9 g NaCl and 1 g peptone in 1000 mL DW. Occasionally, phosphate buffer saline (PBS) solution was likewise used for storing encapsulated/unencapsulated probiotic bacteria, which was prepared by dissolving 4 g NaCl, 0.1 g KCl, 0.575 g $Na_2HPO_4 \cdot 2\ H_2O$, 0.1 g KH_2PO_4 in 500 mL DW. Both solutions were sterilised in autoclave (at 121°C for 15 min) before their use.

3.2.4 Phosphate buffer

0.1 M phosphate buffer was applied to dissolve gel capsules and thereby release encapsulated probiotics for further enumeration. The pH of this buffer solution necessarily varied between 6.8 and 7.5, depending on the specific polymer type of gel matrices to be dissolved. This buffer solution was principally prepared by mixing 0.2 M NaH_2PO_4 and 0.2 M Na_2HPO_4 stock solutions in a specific volume ratio that resulted the desired final pH for the buffer solution. This solution was then sterilised in autoclave (at 121°C for 15 min).

3.2.5 Applied food matrices

In the study of storage stability of probiotic cells, commercial organic beetroot juice (Steinberger) and oat drink (enerBio) were purchased and applied as model non-dairy food matrices. Some criteria were considered for the selection of non-dairy food products, such as the high degree of purity and no (or very minimal) antimicrobial content. Since both products were readily acquired in pasteurised form, no additional sterilisation step needed to be performed.

3.2.6 Applied carrier materials (encapsulant agents) for the encapsulation of probiotics

- Sodium alginate (alginic acid) (Sigma Aldrich)
- Gellan gum (Phytagel) (Sigma Aldrich)
- Xanthan gum (Sigma Aldrich)
- κ-Carrageenan (Sigma Aldrich)
- Locust bean gum (Sigma Aldrich)
- Resistant starch (National Starch Food Innovation, UK)
- Lactulose (PanReac AppliChem, Germany)
- Lactosucrose LS40 (Ensuiko Sugar Refining Co., Japan)
- Lactosucrose LS55 (Ensuiko Sugar Refining Co., Japan)
- Chitosan (low molecular weight; Sigma Aldrich)
- DEAE Sephadex A50 (Pharmacia Fine Chemicals, Sweden)
- Carboxymethyl cellulose (Sigma Aldrich)

3.3 APPLIED METHODOLOGIES

All the microbiological-related formulations and investigations mentioned in this present section were carefully carried out under aseptic conditions (by e.g., working under laminar flow and/or using a Bunsen burner around the work area, using sterile tools and media, performing other standard and basic aseptic techniques and good hygiene practice). For sterilising the laboratory tools, including the glass- and metalware ones, either autoclaving (121°C, 15 min) or hot air drying (160°C, 1.5-2 h) was used.

3.3.1 Bacterial culture preparation and its maintenance

Bacterial culture of each strain was prepared using basically the same procedure, differing only in the condition and the time used for the cultivations (to reach the maximum exponential growth rate). The incubation took place in a thermostat cabinet (or room), adjusted to a proper temperature.

To grow *L. casei* 01 into a fresh batch culture, about 0.1 g of its lyophilised DVS preparation was first added to 10 mL MRS liquid medium (see Section 3.2.1), which was then incubated at 37°C for 16-24 h. The reactivation of *L. plantarum* NCDO 1752 differed in that the MRS broth was inoculated with a small amount of bacteria scrapped from the stock cultures on MRS agar slant surface.

In case of *B. lactis* Bb-12, its DVS bacterial culture was anaerobically incubated in TPY liquid medium (see Section 3.2.1) at 37°C for 48-72 h. This anaerobic incubation was ensured by using a GasPak™ jar system with the inoculated culture medium tightly enclosed inside. All types of the culture were kept at 4°C in a refrigerator shortly before their further usage.

3.3.2 Determination of viable bacterial counts

Viable bacterial cell counts in CFU (Colony Forming Unit) per mL or g were enumerated with either by pour plating (Sanders, 2012) or surface drop plating technique (Miles et al., 1938), latter of which was especially needed when a very large size of samples had to be examined at once. *L. casei* 01 and *L. plantarum* NCDO 1752 were enumerated in MRS agar after incubation at 37°C for 24 - 48 h, while *B. lactis* Bb-12 was enumerated in TPY agar after incubation at 37°C for 48-72h under anaerobic condition (see Section 3.3.1). For the serial dilutions, 0.85 % saline solution was used.

For determining viable bacterial cells encapsulated in gel capsules, these capsules were necessarily agitated in 0.1 M phosphate buffer until their complete gel disintegration (~ 15 - 60 min), right before the plating processes.

3.3.3 Applied techniques for encapsulation of probiotic bacteria

The model probiotics were attempted to encapsulate with several techniques, by which the formation of the capsules was based on such principles like the extrusion, emulsification, layer-by-layer deposition and electrospraying of encapsulation agents. For some cases, an additional polymer coating was also applied on the prepared capsules.

Prior to all the encapsulation processes, the previously grown bacterial culture suspension (see Section 3.3.1) was harvested by centrifugation at 10 000 rpm, 4°C for 10 min, followed by double washing of the cell pellet for removing the growth medium residues and resuspension in 0.1% (w/v) peptone water (or PBS). This final cell suspension was stored at 4°C (and with anaerobic condition assured for the bifidobacterial strain) in a refrigerator before being used for the encapsulation purposes.

3.3.3.1 Extrusion method

The extrusion-based encapsulation was basically carried out as described earlier by Krasaekoopt et al. (2004).

- Initially, the cell suspension was mixed with hydrogel solution in a volume ratio of 1:9, using a magnetic stirrer.
- With the help of a syringe, this mixture was extruded dropwise through a needle (0.7 mm of outer diameter, 22G x 8/8 in) into a proper oppositely charged ion contained cross-linking solution.
- After using up the mixture, the resultant gel droplets were kept stirred in the cross-linking solution for 30 min, in order to stabilising their grid structure established with the ionic cross-linking.

3.3.3.2 Emulsification method

Encapsulation of bacteria with emulsification technique was undertaken by following the procedure described in the paper of Sheu and Marshall (1993):

- Cell suspension was mixed in a polymer-based hydrogel solution (100 mL) in a volume ratio of 1:9, using a magnetic stirrer
- The hydrogel solution was gently dripped into a larger amount of sunflower oil (150 mL) containing 0.2% (v/v) Tween 80, while it was stirred continuously at 200 rpm until the formation of a stable W/O emulsion system.
- A 200 – 250 mL of appropriate cationic cross-linking solution was poured gently (along the wall of the beaker) into the polymer-oil emulsion system to achieve their phase separation; during this separation, a formation of gel particle mass was simultaneously started. In this gel formation, stirring was not applied as it could disrupt the proper phase separation.
- Necessary rest of the phase-separated system was followed until all the possible gel particles were formed and settled down at the bottom of the beaker (in the aqueous phase).
- After removing the oil phase with an automatic pipette, the resultant gel particle mass was double washed with and stored (4°C) in 0.1 % (w/v) peptone water.

3.3.3.3 Layer-by-layer self-assembly method

Layer-by-layer self-assembly-based encapsulation involved alternating electrostatic deposition of two oppositely charged carboxymethyl cellulose (-) (CMC) and chitosan (+) (CHI) polyelectrolytes on the surface of the bacteria. This encapsulation was conducted by using a procedure adapted from Diaspro et al. (2002), but with applying 1 % (w/v) CMC and 1% (w/v) CHI solutions as the two polyelectrolyte (coating) solutions. Briefly, the following steps were performed for coating (i.e. encapsulating) the bacteria:

- First, bacterial culture suspension (prepared as in Section 3.3.1) was centrifuged (10 000 rpm, 4°C, 10 min) and the resultant cell pellet was washed.
- To deposit the first polyelectrolyte layer on the bacteria surface, the initial bacterial pellet was thoroughly agitated in one of the polyelectrolyte solutions for 7.5 min at 37°C.
- After the deposition of the first layer, excess polyelectrolyte solution was completely removed by centrifugation (10 000 rpm, 4°C, 10 min) and the cell pellet was double washed with 0.5 M NaCl.
- Afterwards, the second polyelectrolyte layer was adsorbed by agitating this cell pellet in the other polyelectrolyte solution under the same conditions as the previous one.
- Centrifugation and washing were again performed as described above
- The above alternating deposition process of two polyelectrolytes was repeated until the desired number of layer was formed.

The resultant coated bacterial formulations were stored at 4°C before their use and investigation.

3.3.3.4 Electrospraying method

Electrospray-based probiotic encapsulation was carried out using a commercially available Spraybase® instrument (Avectas Ltd., Ireland). The schematic diagram of the whole system is demonstrated in Figure 6, alongside with the applied parameters for the particle formation and bacterial encapsulation. It should be noted that this part of microencapsulation and the related evaluations were fully performed at the Reading School of Pharmacy and the Department of Food and Nutritional Sciences, University of Reading, UK.

The following procedure was done for the electrospray-based encapsulation:

- A low concentrated feed hydrogel solution mixed with the cell suspension (in the volume ratio of 9 : 1) was pneumatically driven through a plastic tubing, towards the electrified blunt needle (19G). The applied pressurised air flow (1 bar) was generated with an air compressor system.
- To induce atomisation (electrospraying) of the hydrogel solution, a high voltage of 7-12 kV was applied to the emitter needle. This voltage range was chosen to prevent the potential bacterial damages, while the electrospraying can be still generated.
- A metal dish containing cross-linking solution bath, which was connected to the oppositely charged rod, was used for the collection and gel formation of the atomised droplets. The distance between the needle tip and the collector dish was 10 cm.
- The particles were then left in the cross-linking solution for 30 min for further solidification.
- The resulted gel particles were centrifuged (10 000 rpm, 4 °C, 10 min) and double washed with sterile PBS.

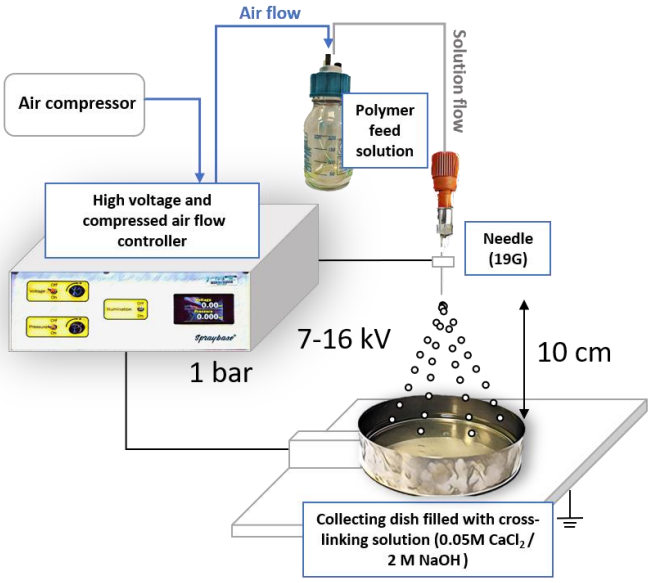


Figure 6. Schematic diagram of the electrospray system applied in the present work

The storage of these formed particles was done at 4°C in sterile PBS. In the later experiments, these particles were examined in semi-drained form with a help of sterile syringe (with 30G needle).

It should be noted that unloaded (without bacterial cells) pure chitosan capsules applied exclusively for the mucoadhesion analysis were prepared by electrospraying 2 % (w/v) chitosan solution (in 0.1 M acetic acid) at 16 kV into a 2 M NaOH-contained bath.

3.3.3.5 Coating capsules with polymer layer

Coating was conducted based on the electrostatic deposition of the applied polymer to a counter ionic capsule surface. For coating negative-charged capsules with positive-charged chitosan, the procedure outlined by Krasaekoopt at el. (2004) was applied with slight modifications. Briefly, 45 g of negative-charged (e.g. alginate) gel capsules were immersed in 300 mL chitosan coating solution (0.4 % (w/v) in 0.1 M acetic acid, adjusted to pH = 6, microfiltered, autoclaved), and were agitated therein using an orbital shaker (100-150 rpm, 30-60 min, 26-28°C). In the case of tinier sized capsules (see Section 4.4.1), 0.5 g capsules were immersed in 10 mL chitosan solution (0.2 % (w/v) in 0.1 M acetic acid, adjusted to pH = 6, microfiltered, autoclaved).

For coating with positive-charged DEAE Sephadex A 50 (by carrying a cationic group), a 1 % (w/v) solution was prepared and used up for the coating in the same way as with chitosan.

3.3.4 Applied methods for physical characterisation of the capsules

3.3.4.1 Morphology evaluation and imaging of the capsules

For the microscopic observation and imaging of the resultant capsules, several types of microscope and related tools (e.g., camera, software) were applied for different types of capsule.

- Microscopic imaging and analyses of multiple polyelectrolyte coated bacteria were carried out with an **inverted microscopy** (Nikon Eclipse Ti-E), featuring with phase contrast and fluorescence modules. The microscopic analysis was controlled through NIS-Element imaging software and the samples were captured using an Andor NEO sCMOS camera. This specific microscopy study was conducted at Biological Research Centre of Hungarian Academy of Sciences in Szeged.
- The resultant electrosprayed particles were imaged and analysed with a **fluorescence microscope** (Leica MZ10F) using an ET-GFP filter. For their detection, sodium fluorescein (Na-Fluo) and fluorescein isothiocyanate (FITC) - labelling was employed for alginate and chitosan related samples, respectively. For acquisition of these fluorescent images the light level with exposure time of 57 ms was used for sodium fluorescein-labelled and of 100 ms was used for FITC-labelled samples, and with the pseudo color wavelength of 520 nm and a slight black/white level

correction adjusted. ImageJ software (version 1.52a, USA) was used further quantitative evaluations in terms of fluorescent pixel intensity. Microscopic images of particles were additionally obtained from **Malvern Morphologi 4** automated image characteriser which was featured in a **Metasizer system** (See Section 3.3.4.2).

3.3.4.2 Measurement of dimension and size distribution of the capsules

The dimension and size distribution of the capsules were measured with using a **caliper**.

For tiny sized capsule particles, these were determined with a **laser light diffraction analysis** using **Metasizer 3000 instrument** (Malvern Instruments, Malvern, UK). This instrument was equipped with a Hydro Medium Automated Volume dispersion unit for the controlled, automated wet dispersion of samples during the analysis. For the particle size analyses with the Metasizer apparatus, the samples were prepared with particles suspended in some purified water [10 % (w/v)]. Particle refractive index, dispersant refractive index and absorption index value were chosen as 1.4, 1.33 and 0.1, respectively. This particular size measurement was conducted in Reading, UK.

The resulted particle size distributions were characterised for the volume-weighted mean diameter (D (4,3)) and the width of the size distribution (Span) according to the following formula (ASTM Standard E799-03, 2015; Resch-Genger, 2008):

$$D\ (4,3) = \frac{\sum d_i^4 \cdot n_i}{\sum d_i^3 \cdot n_i}$$

$$\text{Span} = \frac{d_{v0.9} - d_{v0.1}}{d_{v0.5}}$$

where d_i is the diameter and n_i is the number of the i-th particles; $d_{v0.9}$, $d_{v0.1}$ and $d_{v0.5}$ (median) represents the diameter, below which the 90%, 10% and 50% of the population lies, respectively. Some dimensional measurements were also carried out with using ImageJ software.

3.3.4.3 Textural analysis of the gel capsules

Texture measurements were carried out with applying Brookfield LFRA 4500 Texture Analyzer. Two types of compression cycle were run aiming to acquire two different texture profiles, namely about springiness and mechanical strength for each capsule type. For this, a non-destructive test for the former and a destructive test for the latter texture profile were conducted on the same batch particle sample. A batch sample (of each capsule type) was made up of 10 randomly selected individual particles and was measured in 9 replicates (in 9 separate batches). From the data related to the

springiness and mechanical strength profile curves, several physical parameters can be extracted, such as the hardness (the peak force in each compression run), the area cycle (the area covered by the curve obtained between 0 min and the timepoint of the peak force), the adhesive force (detachment (negative) force from the compressing probe; also partially reflecting about the solidness) and the fracture force (brittleness; the force with which the first significant rupture of the particles occurred during the compression run). All of these parameters are further defined by Szczesniak (2002), Razavi and Karazhiyan (2012). In addition, springiness was determined based on the hardness value obtained with the non-destructive test. In case of the non-destructive test, the fracture force parameter was ignored. The applied compression length was 1.0 mm for the non-destructive and 2.0 mm for the destructive run, while the compression speed was equally set to 0.1 mm/s.

3.3.4.4 Mucoadhesion test of the capsules

To examine mucoadhesive ability of the microcapsules on mucosal tissue, an *in vitro* fluorescence flow-through retention (wash-off) test was undertaken by modifying the traditional procedure applied by Cook et al. (2018), Kaldybekov et al. (2018) and Porfiryeva et al. (2019). In this study an *ex vivo* porcine gastric tissue was used as a model mucosal membrane. Retention on mucosal surface depends on the mucoadhesive strength of the microcapsules while continuously being washed off with simulated gastric fluid; this retention was monitored and investigated through the microscopic imaging of the fluorescently labelled microcapsules on the mucosal surface at regular time intervals.

The main modification made in my study was that the retention was observed under a 1080P 1000X Zoom HD 8LED Digital USB Microscope Magnifier Endoscope Video Camera. In this case, a Winzwon UV torch was used as an external light source to illuminate and detect the fluorescently-labelled microparticles, whereas the internal light source of the microscope itself was switched off for the whole experiment to increase the fluorescent intensity. The AmCap ver. 9.0 software was used for recording the images of the samples. The retention studies were conducted using the experimental set-up shown in Figure 7.

Porcine stomachs were collected from an abattoir (North Camp, UK) in a cold storage box (~ 4°C). The stomach was dissected, then smaller and smooth rectangular tissue pieces (approximately 1 × 1.5 cm) were carefully ablated from the mucosal fold part (rugae) of the stomach lining, using a surgical scalper. Simulated gastric fluid (SGF) pre-warmed in a 37°C water bath was used for modelling the wash-off process of the test microparticles from the mucosal surface. The bare mucosal tissue surface

– i.e., without any test microparticles fixed thereon – was pre-rinsed right before the wash-off test, and the mucin monomers on this examined mucosal surface were present in cross-linked (hydrated gel) form.

Experimental set-up

The mucoadhesion test was conducted with an own assembled experimental set-up illustrated in Figure 7. By using lab stands, a microscopic slide was fixed at an angle of 20 ° to the ground to ensure the consistent flow of simulated gastric fluid through the microparticles, while the portable microscope was positioned perpendicularly to the tissue surface, pointing the objective lens in the direction of particle mass on the mucosal tissue. The UV torch lamp was placed at an angle of 45° and at a distance of 40 mm from the tissue surface. With this arrangement, the aim was to ensure that the UV light intensity provides an optimally exposed and threshold imaging of the fluorescent particles (distinguishing them from the background fluorescence).

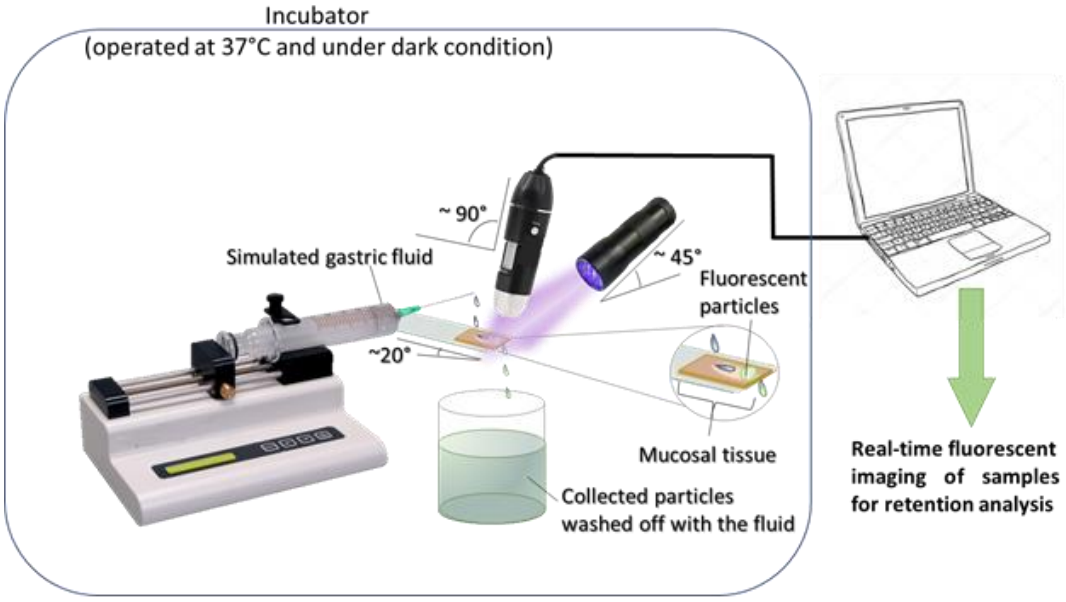


Figure 7. Illustration of the assembled experimental set-up for the retention study of particles on gastric mucosa

The retention (wash-off) test was performed in an incubator chamber at 37°C under dark conditions, and in the following steps:

- Prior to the wash-off process, the tissue piece and the SGF were preconditioned at 37°C in the incubator and in a water bath, respectively.

- An aliquot (0.02 g) of fluorescent-labelled particles was spread over one of the edge area of pre--rinsed mucosal surface of the tissue piece. This tissue piece was fixed on the edge of a microscopic slide.
- After the particles were applied on the mucosal surface, the preconditioned (37°C) SGF was dripped from a needle onto the mucosal surface using a digital syringe pump at a constant flow rate of 5 ml/min. This specific flow rate was used to mimics the average *in vivo* gastric secretion rates reported for both fasted and active digestion periods (Versantvoort et al., 2004). The series of droplets was directed to fall from a height of 15 mm to exclude the needle from the microscope field of view, and around 5 mm away from the mucoadhesive formulation to ensure efficient wash-off process. All the fluid passed through the mucosal tissue was simultaneously collected in a container.
- The target tissue area of interest was captured using the camera of the microscope at specific time points, after interrupting the washing process and the liquid was totally drained off (~ 2 min). In order to capture the entire particle mass on the tissue, I used a 40x magnification on the microscope and a distance of around 15 mm between the objective lens and the tissue surface (particle mass) during the whole test. All the acquired images underwent the same light intensity correction and were analysed using ImageJ software to quantify the intensity of fluorescence after each period of wash.

All these experiments were performed in triplicate for each formulation using an incubator at 37°C and under dark conditions.

3.3.5 Applied methods for physiological evaluation of encapsulated (and free) bacteria

3.3.5.1 Determination of encapsulation yield

Encapsulation yield indicates the percentage of the bacterial mass that survived the particular encapsulation process. This value was calculated using the formula below (Haghshenas et al., 2015):

$$\text{Encapsulation yield } (\%) = \frac{\log_{10}(\text{N})}{\log_{10}(\text{No})} \cdot 100$$

where N is the viable number of encapsulated cells released from the resultant capsules and No is a total number of viable free cells added initially to polymer solution and used up for their encapsulation. To determine the N value, entrapped bacteria were fully released by agitating (15-60 min, 1000 rpm)

the capsules in 0.1 M phosphate buffer (pH=7.5), then the released bacteria were enumerated using plating method as in Section 3.3.2.

3.3.5.2 Survival test in simulated gastrointestinal fluids

The survival test was carried out based on the widespread static procedure described earlier by Krasaekoopt et al. (2004), with some modifications. These survival tests involved sequential treatments in gastric and intestinal (duodenum) phases, during which the samples were subjected to a highly acidic and a subsequent bile salt condition, respectively. The applied artificial gastric solution (SGF) composed of 0.2 % (w/v) NaCl, with adjusted to pH = 2 using HCl. The simulated intestinal solution (SIF) contained 0.05 M KH_2PO_4, which pH was adjusted to the value of 7.43. As is mentioned before, all these media were sterilised prior to the survival assays. The addition of pepsin [0.3 % (w/v)] (Sigma Aldrich) into SGF and porcine bile extract [0.6 % (w/v)] (Sigma Aldrich) into SIF was always applied after the autoclaving.

Gastric phase

In this part, cell-loaded capsules / free cells were digested in separate runs for different exposure time-lengths, including 45, 90 and 135 min. For this purpose, the following steps were done:

- 0.5 g of cell-loaded capsule or 0.5 mL free cell sample was placed in a test tube, into which 5 / 4.5 mL SGF (pH = 2) was measured.
- The test tube was placed and kept in an incubator at 37°C for 45 min / 90 min / 135 min.
- After the end of each target incubation time, SGF was immediately discarded from the test tube with macro pipette. In the case of free cells, a prior centrifugation was necessarily used for discarding SGF (as a supernatant form).
- The survived bacterial cell counts were determined as in Section 3.3.2.

Intestinal phase

After the gastric phase, the sample was exposed to the intestinal medium as below:

- The test tube with removed gastric solution was filled with 5 mL of SIF (pH = 7.43).
- The test tube was then put and kept in an incubator at 37°C for 150 min.
- After the end of the target incubation time SIF was immediately discarded from the test tube by macro pipette aspiration

- The survived bacterial cell counts were determined as in Section 3.3.2.

The survival test with **electrosprayed microcapsules of much tinier size** was carried out using a slightly modified procedure described above. The following modifications were applied: (1) the ratio of the sample (microcapsules / free cells) to the digestion fluid was different, (2) only gastric phase was applied, and (3) the digestion was conducted for only two time-lengths (1h and 2 h, in separated runs). Thus, this simulated digestion was performed as outlined below:

- 0.05 g bacteria-loaded microparticle / 0.1 mL of the free cell suspension was suspended in 0.9 mL SGF.
- The sample was then incubated for 1 h / 2 h at 37°C.
- After the end of the incubation, SGF was discarded with immediate centrifugation (10 000 rpm, 4°C, 10 min).
- In case of the sample with microcapsules, bacteria were released therefrom by resuspending and agitating (1000 rpm, 15 min) in 1 mL 0.1 M phosphate buffer (pH=7.5) until their complete disintegration.
- Survived bacterial number of any type of sample was enumerated using the method described in Section 3.3.2.

3.3.5.3 Simulated digestion based on a sophisticated and harmonised Infogest protocol

Digestion study was also carried out according to the static *in vitro* protocol developed by an international consensus within the COST Infogest network, which harmonises other several *in vitro* protocols reported for simulating human digestion. This complete digestion protocol was followed as described precisely by Minekus et al. (2014). In this case, the applied composition of simulated gastric and intestinal fluids (SGF and SIF) highly differed from the previously mentioned *in vitro* protocol (Section 3.3.5.2), regarding their more complex electrolyte content and enzyme activity. Furthermore, an additional simulated salivary fluid (SSF) was also included for representing the oral phase.

As is required in this protocol, preliminary activity and concentration assays were done for each digestive enzyme and bile acid preparation (Table 4) in order to meet the activities / concentrations required for final digestion fluids. These assays were carried out based on the recommended methods given in the supplementary material for the paper of Minekus et al. (2014).

In the case of pancreatin enzyme preparation, the amount of its stock solution added to final SIF mixture was based on the trypsin activity, while the activity of other enzyme components (like chymotrypsin, α-amylase, lipase) was measured for obtaining supplementary information.

It should be noted that a slight modification was necessarily made with regard to the applied origin of the amylase enzyme, differing from that recommended for this protocol. This modification is detailed in Table 4. Although, since the respective enzyme was added based on its activity to the reaction vessel, the results of this digestion can be still comparable with any other results obtained by the Infogest method.

Table 4. Enumeration and characterisation of the enzymes and bile used in Infogest digestion model during the present work

Reagent recommended by Minekus et al. (2014)	Reagent used in the present work	Enzyme activity/ bile acid content [1]
Human salivary α-amylase	Sigma-Aldrich 10080: α-amylase from hog pancreas	37.9 U/mg amylase activity
Porcine pepsin	Sigma-Aldrich 77161: pepsin from porcine gastric mucosa	88.4 U/mg pepsin activity
Porcine pancreatin	Sigma-Aldrich P1750: pancreatin from porcine pancreas	3.17 U/mg trypsin activity 1.2 U/mg chymotrypsin activity 56.17 U/mg amylase activity 112.22 U/mg lipase activity
Sigma-Aldrich B8631 (porcine) or B3883 (bovine) or fresh (frozen) porcine bile	Sigma-Aldrich B3883: bile bovine	0.876 μmol/mg bile acid

[1] Determined in the applied corresponding enzyme / bile preparations

Before the whole Infogest digestion process, all the prepared, not final SSF, SGF and SIF solutions – i.e. with electrolyte content, but without enzyme and bile addition and pH adjustment – were stored at 4°C and preconditioned at 37°C. The bacteria counts were enumerated after the simulated gastric and intestinal digestion phase, as described previously in Section 3.3.2.

3.3.5.4 Heat-treatment

High-temperature treatments at 60°C and 85°C of the microencapsulated and free bacteria were performed. This treatment was repeated for different exposure times, for which the following approach was undertaken:

- In case of the encapsulated bacteria, 0.5 g of the capsules was suspended in a test tube containing 1 mL 0.1 % (w/v) peptone water.
- The test tube was immersed in a water bath set to 60°C / 85°C and left therein for 2 / 5 / 10 / 20 min.
- To terminate the heat treatment, the sample was gently chilled down by immediate transferring and dipping the test tube for a short time in an ice water bath
- Viable bacterial number were subsequently determined as detailed in Section 3.3.2.

In this experimentation, peptone water [0.1% (w/v)] was used as the model food matrix.

3.3.5.5 Storage experiment in a food matrix

To do storage experiments, the following steps were performed:

- Firstly, 200 mL food medium in a flask was inoculated with 20 g of encapsulated cell / 2 ml of free cell sample.
- The fermentation was initiated in a thermostat incubator at 37°C for 24h.
- After this fermentation step, 200 mL food medium containing the sample was distributed evenly into two separate flasks.
- One part of the sample was then kept in refrigerator at 4°C, while the other one was stored outside at 20°C.

3.3.6 Compositional analysis of carbohydrate and acid content in food media

The quantitative contents of carbohydrates and organic acids were analysed with HPLC (High Performance Liquid Chromatography). HPLC testing were conducted on a Thermo Scientific Corporation Surveyor instrument equipped with four-channel LC pump (Surveyor) and polystyrene-divinylbenzene column (Aminex HPX-87H, 300 x 7.8 mm, Bio-Rad) and 0.005 N H_2SO_4 was used as mobile phase. For detecting and measuring the amount of various carbohydrate and organic acid components, RI and PDA detectors (Surveyor) were applied at 410 nm and 210 nm, respectively. Flow rate of 0.6 mL/min and loop of 10 µl were used in every case.

External standards were applied for the determination of carbohydrate and organic acid concentration. (Carbohydrate standards: glucose, maltose and saccharose; Organic acid standards: lactic and butyric acid)

3.3.7 Preparation of fluorescently labelled microcapsules

Fluorescently labelled alginate and resistant starch-alginate microcapsules were prepared by electrospraying polymer solutions containing 0.1% (w/v) Na-Fluo; in the case of the mucoadhesion study, 0.1% (w/v) FITC-dextran was used. Chitosan polymer – used as coating – was labelled with FITC using the protocol described in a previous study (Cook et al., 2011). Chitosan-based particles used in the mucoadhesion study were prepared from chitosan solution labelled with 0.1 % (w/v) FITC.

3.3.8 Statistical analysis

Data were analysed statistically with either SPSS (Statistical Package for the Social Sciences, ver. 25.0., USA) or GraphPad Prism software (version 8.0; USA). ANOVA (analysis of variance) with a significance level of $\alpha = 0.05$ was used to determine statistical differences among the independent variables. For multiple comparisons of these ANOVA results either Tukey's or Games-Howell post-hoc test was applied depending on the given particular dataset – as to whether it meets the homogeneity of variance test. Most of the final data were reported as average value of replicates, along with their corresponding standard deviations.

Hierarchical agglomerative cluster analysis was used in the textural-related studies for identifying significant differences among the samples in the set of textural characteristics. Significance groups were derived based on the function of squared Euclidean distance.

4 RESULTS AND DISCUSSION

4.1 PHYSICAL AND PHYSIOLOGICAL EVALUATION OF ENCAPSULATED PROBIOTICS

By utilising probiotics in microencapsulated form, an enhanced efficiency of health-promoting functions can potentially be achieved for probiotic foods. However, we should find out what particular microencapsulation approaches are powerful to generate capsule systems that suitable for proper probiotic delivery and incorporation in real food matrices. First of all, morphological and textural characteristics of these capsules are important aspects when it comes to incorporating in and consuming them through the probiotic food products, considering the possible unfavourable changes in the sensory characteristics thereof. Furthermore, the capsule systems should ideally be effective in the protection of their microencapsulated probiotic viability, with maintaining the viable cell counts above the suggested minimal level (i.e., 6 log CFU/g or mL in small intestine and 8 log CFU/g in colon) for ensuring their therapeutic functions after the delivery through several harsh environmental factors (e.g., strong gastric acid and bile salt condition, heat-treatment, possible acid sensitivity and oxygen toxification upon long-term storage in food matrices). Accordingly, the loading capacity of the capsules with viable probiotic cells should be as high as possible. Besides viability protection, a sufficiently prolonged gastrointestinal retention of probiotics has also emerged as another crucial factor for supporting the effective gastrointestinal delivery of these microorganisms. Therefore, it is also important to evaluate the mucoadhesion characteristic of probiotic-loaded formulations.

4.2 EVALUATION OF THE PROBIOTIC-LOADED CAPSULES PREPARED BY EXTRUSION TECHNIQUE

4.2.1 Preparation of extrusion-formed capsules

In this work, the first encapsulation was performed with extrusion technique and with model probiotic *Lactobacillus casei* 01. Accordingly, my aim was to prepare different polymeric variations of the capsules with this extrusion technique, as a result of which the following variations were successfully formed: calcium alginate (referred later as just ‘alginate’), calcium alginate combined with a prebiotic component like resistant starch, lactulose, lactosucrose LS40L and lactosucrose LS55L; and non-alginate capsules like blends of gellan gum-xanthan gum and κ-carrageenan - locust bean gum. The study of these specific types of the capsules was particularly inspired by that alginate has been one of the most commonly used biopolymers for constructing probiotic-loaded capsules so far – by offering several technological benefits e.g., high biocompatibility, low cost. However, alginate-based capsules

have also been reported to provide weak bacteria protection against stress environmental factors due to the high porosity (~ 17 nm) of their gel matrix and for the sensitivity of their gel matrix to the presence of anti-gelling cationic agents like Na^+-ions (typical in e.g., digestion fluids) (Chavarri et al., 2012; Chen and Chen, 2007; Cook et al., 2012; Gombotz and Wee, 2012; Martín et al., 2015; Smidsrød and Skjåk-Bræk, 1990). Table 5 presents all the obtained capsule variations, along with the applied exact compositions of hydrogel and cross-linking solutions needed for their successful formation. Moreover, the alginate capsules were additionally coated with chitosan and DEAE Sephadex A 50 polymer layer (Table 6) in order to reveal the effect of these polymer coating on the probiotic protection ability of alginate capsules compared to that of the uncoated ones. All these capsule variations were subsequently investigated for their morphology, size distribution, textural characteristics, encapsulation yield and probiotic protection ability under simulated digestion conditions.

Table 5. Different variations of successfully prepared gel capsules, and the composition of hydrogel and cross-linking solutions used for their extrusion-based preparation.

Types of the capsules	Composition of hydrogel solution		Cross-linking solution
	Component 1	Component 2	
Pure alginate	-	2% (w/v) Alginate	0.05 M $CaCl_2$
Alginate combined with prebiotics	2% (w/v) Resistant starch	2% (w/v) Alginate	0.05 M $CaCl_2$
	2% (w/v) Lactosucrose LS55L	2% (w/v) Alginate	0.05 M $CaCl_2$
	2% (w/v) Lactosucrose LS40L	2% (w/v) Alginate	0.05 M $CaCl_2$
	2% (w/v) Lactulose	2% (w/v) Alginate	0.05 M $CaCl_2$
No alginate inclusion	0.75% (w/v) Gellan gum	1% (w/v) Xanthan gum	0.1 M $CaCl_2$
	2% (w/v) κ-carrageenan	1% (w/v) Locust bean gum	0.3 M KCl

Table 6. Different variations of successfully coated calcium alginate capsules and the applied solutions for their coating process

Types of the capsules	Polymer coating solution	Capsule matrix (hydrogel solution)
Chitosan coated alginate	0.4% (w/v) Chitosan	2% (w/v) Alginate
DEAE Sephadex coated alginate	1% (w/v) DEAE Sephadex A-50	2% (w/v) Alginate

4.2.2 Morphology of the extrusion-formed capsules

Photographs were taken of all variations of extrusion-formed gel capsules, which are shown in Figure 8. Shape of regular sphere (bead) could be formed in case of alginate-, resistant starch-alginate-

(Figure 8A and B) and every coated alginate-based gel formulation, whereas the shapes of lactulose-alginate, lactosucrose LS40L-alginate, lactosucrose LS55L-alginate, κ-carrageenan-locust bean gum and gellan gum-xanthan gum (Figure 8C - H) capsules were rather irregular. In case of the former three varieties, this can be attributed to the fact that these prebiotic components could interfere the ionotropic interaction between alginate and cross-linker Ca^{2+}-ions or decrease the viscosity of alginate (hydrogel solution). As for the non-alginate hydrogels, having a higher optimal gel-melting temperature point than the highest temperature sensitive that the bacteria can tolerate made the extrusion through the needle more complicated – i.e. the needle got clogged by too early congealment of the gel during its passage therethrough. It also appeared that the spherical shape observed for alginate beads was not distorted after coating with either chitosan or DEAE Sephadex.

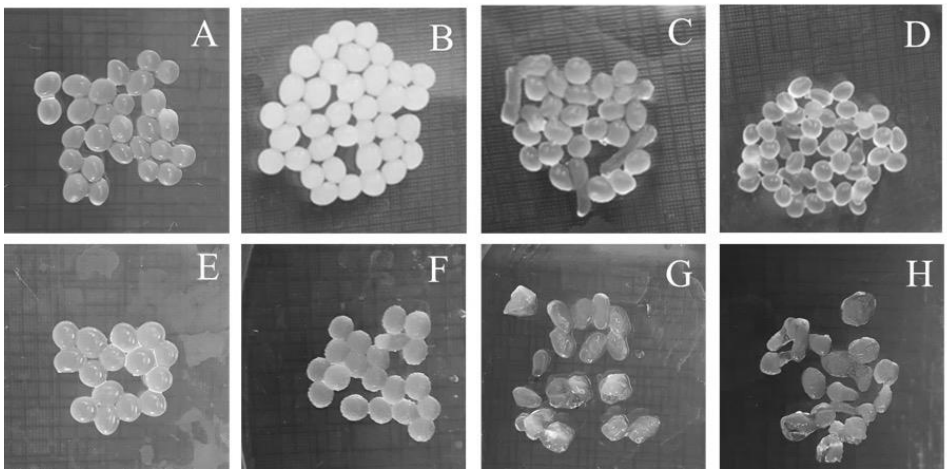


Figure 8. Appearance and morphology of calcium alginate (A), resistant starch – alginate (B), lactulose – alginate (C), lactosucrose LS40L / LS55L – alginate (D), chitosan coated alginate (E), DEAE Sephadex A 50 coated alginate (F), gellan gum – xanthan gum (G) and κ - carrageenan – locust bean gum gel capsules (H) prepared with extrusion technique (capsules are not depicted in actual scale)

4.2.3 Size distributions of extrusion-formed capsules

Size of the formed capsules are important aspects when it comes to incorporating them in and consuming them through probiotic food products, given the possible unfavourable changes in the sensory characteristics (e.g., texture, flavour) thereof. The size distribution of each type of extrusion-formed gel capsules was characterised based on a size measurement of multiple random individual capsules using a digital calliper. Size distributions of different gel capsules, along with the mean and the standard deviation values, are presented in Figure 9a-9b.

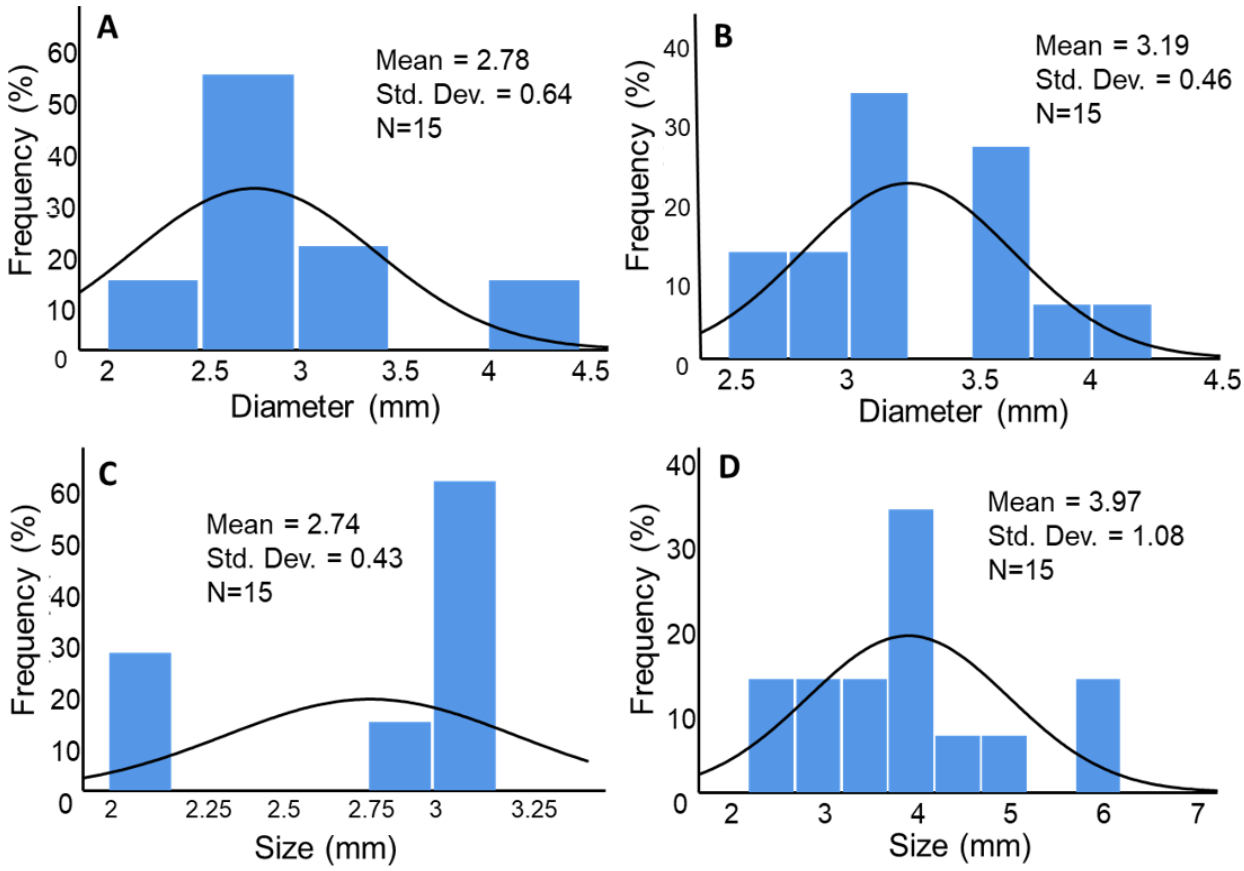


Figure 9a. Particle size/diameter distributions of different extrusion-prepared gel capsules: alginate (A), the blend types of resistant starch – alginate (B), lactulose – alginate (C), lactosucrose LS40L - alginate (D).

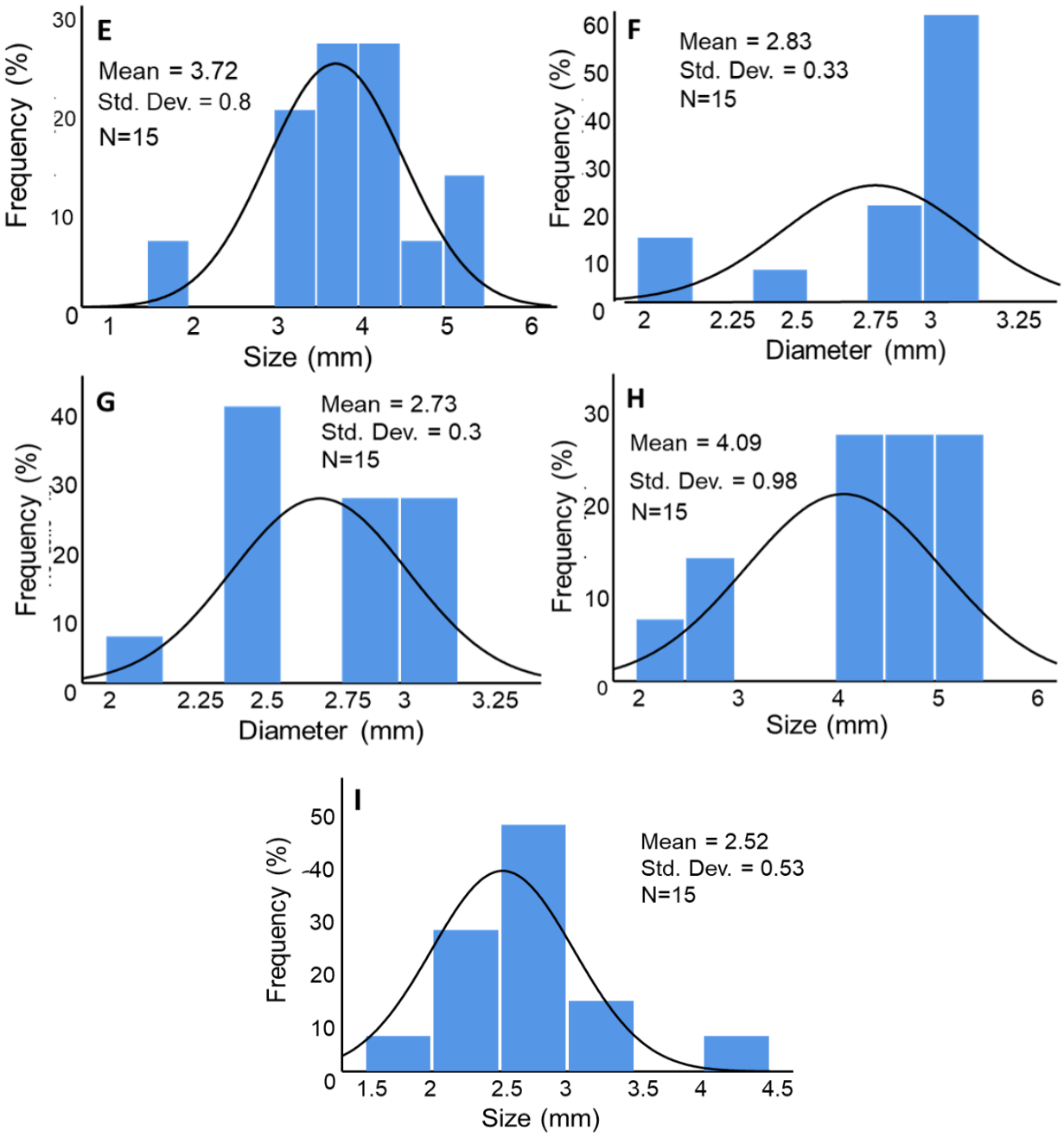


Figure 9b. Particle size/diameter distributions of different extrusion-prepared gel capsules: alginate lactosucrose LS55L - alginate (E), the coated types of chitosan coated alginate (F), DEAE-Sephadex coated alginate (G), and the non-alginate blends of gellan gum – xanthan gum (H) and κ-carrageenan – locust bean gum (I).

Wide size (in case of non-spherical beads) or diameter (in case of spherical beads) distribution lied in the range of millimetre, with varying from 2 mm up to 5.5 mm. High distributions with 3 mm and 4 mm diameter differences were detected. This wide millimetre size range was similarly obtained with

extrusion technique by Krasaekoopt et al. (2004) as well as Argin (2007) and Lee and Heo (2000). Except for the lactulose-blended and the DEAE Sephadex coated ones, all modified variations of prepared alginate-based capsules – including the blend of resistant starch-alginate capsules with an average diameter of 3.19 mm and the chitosan coated alginate capsules with 2.83 mm – were obtained in notably greater size/diameter as compared to that of the pure alginate capsules with 2.78 mm; this increased size/diameter somewhat indicates the successful process of the prebiotic-blending and the chitosan-coating. Krasaekoopt et al. (2004) and Yeung et al. (2016), who prepared gels with the same procedure, also obtained significantly greater beads by coating with several polymers, including chitosan. Muthukumarasamy et al. (2006) achieved nearly the same mean diameter for alginate capsules [with an applied concentration of 3% (w/v)] and a slightly smaller diameter for resistant starch-alginate capsules [2-3% (w/v)]. In my study, the biggest gel capsule was recorded for gellan gum-xanthan gum capsules with an average size of 4.09 mm, while the smallest one was obtained for the κ-carrageenan-locust bean gum -based ones with an average size of 2.52 mm – formed even smaller than the pure alginate ones (2.78 mm). However, with the gel composition of 0.5 - 1% (w/v), Muthukumarasamy et al. (2006) produced gellan gum-xanthan gum capsules in smaller size than alginate [3% (w/v)], resistant starch-alginate [2 - 2% (w/v)] and κ-carrageenan-locust bean gum [1.75 - 0.75% (w/v)] capsules.

4.2.4 Encapsulation yield of extrusion-formed capsules containing *L. casei* 01

Encapsulation yield of live *L. casei* 01 in different variations of the capsules was determined to reveal and compare the effect of different encapsulating agents on the bacterial loading capacity with extrusion method. High encapsulation yield is beneficial for assuring the high viable bacterial number at the time of their consumption (through probiotic foods). As it can be seen in Figure 10, significant differences were detected among the different capsule types in the encapsulation yields of *L. casei* 01 ($p < 0.001$). With alginate capsules, encapsulation of the bacteria was obtained with a yield of 64.4%. However, modification made on these alginate capsules resulted significantly ($p < 0.05$) greater encapsulation yields by averagely 14 % with prebiotic co-constituents and by averagely 9% with external coating. In more specific, encapsulation into resistant starch-alginate, lactulose-alginate, LS44L-ALG, lactosucrose LS55L-alginate yielded bacterial loading of about 77 %, 78.6 %, 78.1 %, 79 %, respectively; coating with chitosan and DEAE Sephadex yielded about 77% and 68.3 %, respectively. Sallehudin et al. (2017) also reported this relatively low encapsulation yield for alginate capsules, while Sultana et al. (2000) also confirmed that incorporation of Hi-Maize starch as prebiotic

improved the encapsulation of probiotics into alginate gel matrix. The low yield obtained with the solely alginate hydrogel may be explained by that a high degree of bacteria leakage could possibly occur from alginate gel matrix to the aqueous cross-linking solution due to the mechanical stirring (~ 200 rpm) applied for promoting the alginate gel solidification. However, the increased recovery of bacterial number came with the incorporation of starch or even other components indicates that the additional presence of carbohydrate granules within the alginate gel cavities might decrease the pore size of alginate gels and thereby provide higher bacteria retentions within the gel matrix during the stirring step.

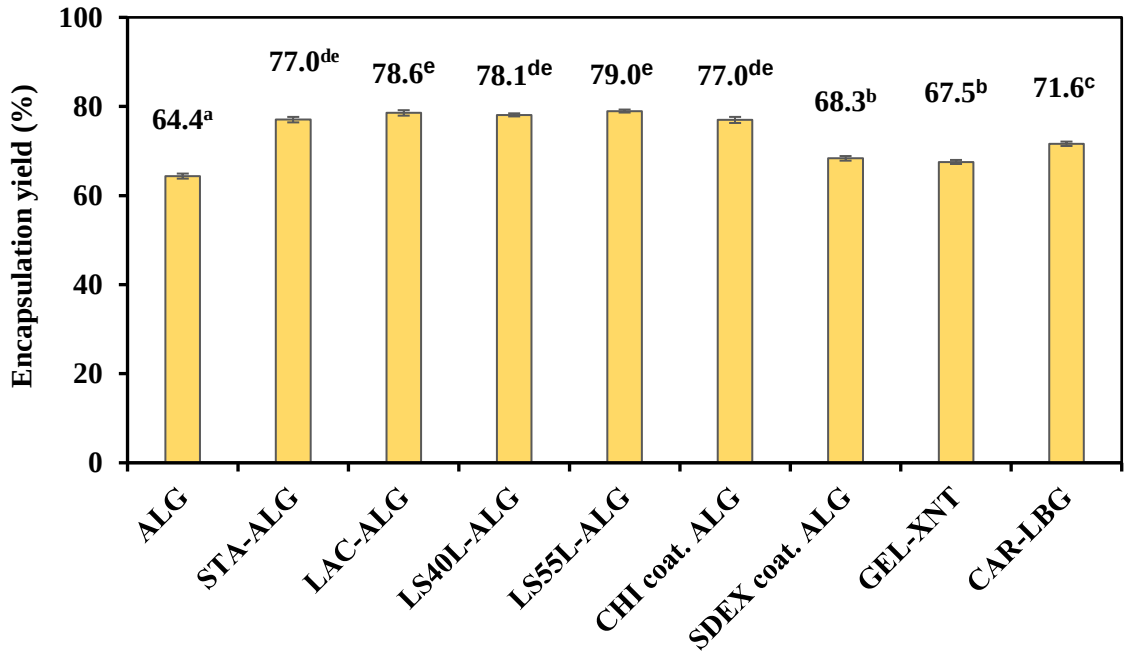


Figure 10. Encapsulation yield of different extrusion-formed gel capsules containing *L. casei* 01. Same letters next to the percentage values indicate non-significantly differences (p > 0.05). Abbreviations: ALG, alginate; STA-ALG, resistant starch – alginate; LAC-ALG, lactulose – alginate; LS40L-ALG, lactosucrose LS40L – alginate; LS55L-ALG, lactosucrose LS55L – alginate; CHI coat. ALG, chitosan coated alginate; SDEX coat. ALG, DEAE Sephadex coated alginate; GEL-XNT, gellan gum – xanthan gum, CAR_LBG, κ-carrageenan – locust bean gum

The encapsulation yield did not vary a lot among the prebiotic included blends with a maximum difference of 2 %, while chitosan coating induced better encapsulation yield for alginate beads than DEAE Sephadex coating by 8%. Krasaekoopt et al. (2004) showed no significant difference regarding to chitosan coating with the same procedure. Moreover, non-alginate types, namely gellan gum-

xanthan gum and κ-carrageenan-locust bean gum capsules also provided significantly better encapsulation of bacteria as compared to the pure alginate ones by 3.1% and 7.2 %. Among them, the higher encapsulation yield of 71.6 % was obtained with κ-carrageenan-locust bean gum, while ~ 68 % with gellan gum-xanthan gum. However, they still provided weaker yield as compared to the prebiotic included and the chitosan coated alginate ones.

4.2.5 Texture profile of extrusion-based capsules

Textural analysis of all the prepared gel particle variations was further performed to assess their firmness, physical stability and expected mouthfeel as delivery systems (food ingredients) when used for human consumption. In addition, texture characteristic was also determined to find out its possible impact on the protection ability of the capsules under harsh acidic and bile salt conditions (see Section 4.2.6). Table 7 compares each textural characteristics of each variety of gel particles retrieved with non-destructive (springiness related) and destructive (mechanical strength related) test. The hardness parameter of both runs varied significantly ($p < 0.001$) among the examined types of gel capsule, whereas the fracture force was significantly invariant. No significant difference adhesive force was only observed in the case of destructive compression run. According to this type of hardness attributes, the modified alginate capsules were mostly significantly comparable ($p > 0.05$) to the control alginate ones, except the ones with chitosan coating layer. Further research should be performed to find the accurate explanation for this observation. However, it could be partially due to the prior roughly 1-hour exposure of alginate gels to the possibly altered pH environment (~ 6) of chitosan solution (see Section 3.3.3.5). This pH may somewhat cause gel swelling (Cook et al., 2011), thereby some mechanical weakening of gel matrix could occur. In the study of Krasaekoopt et al. (2004), the coatings, including the chitosan one, interestingly did not appear to change the mechanical strength for alginate beads. The statistically highest (and nearly equal) hardness values were measured for resistant starch-alginate (453.6 g) and lactosucrose LS55L-alginate capsules (457.6 g). Among the non-alginate capsules, the mechanical characteristics of κ-carrageenan-locust bean gum ones were much closer to the control capsules. On the other hand, the gellan gum-xanthan gum capsules showed the poorest overall mechanical characteristics, considering that the lowest hardness (26.5 g) was observed in their case. Without destruction of the capsules, the adhesion force values indicated that gellan gum-xanthan gum tended to adhere more to the compression probe, while in any other cases this textural attribute was found rather equal among the examined types of the capsules. The good mechanical strength of alginate and resistant starch-alginate capsules were also shown according to

the hardness values of non-destructive test that, at the same time, typically informs about the springiness characteristics, which also reflected the weak strength of gellan gum-xanthan gum capsules.

Table 7. Intensity of texture attributes for each variety of extrusion-formed gel capsules obtained with a texture analyser. Different letters within the row of each attribute represent significantly different means ($p < 0.05$) (mean ± STD, 9 replicates on 10-capsule batch of each capsule type)

Texture profile	Texture attributes	Alginate	Resistant starch - Alginate	Lactosucrose LS40L- Alginate	Lactosucrose LS55L- Alginate	Lactulose - Alginate	Chitosan coated alginate	DEAE Sephadex coated alginate	Gellan gum – Xanthan gum	K-carrageenan – Locust bean gum
Springiness	Hardness (g)	209.3 ± 37.9^{a}	177.1 ± 25.4^{ab}	45,2 ± 16.6^{d}	64.95 ± 34.3^{cde}	121.7 ± 39.2^{bc}	34.4 ± 7.4^{de}	74,1 ± 27.9^{c}	24.6 ± 12.1^{e}	41.0 ± 17.8^{de}
	Area cycle (gs)	854,9 ± 130.4^{a}	901.3 ± 129.1^{a}	207.5 ± 71.0^{d}	325.8 ± 180.3^{bc}	549,3 ± 171.6^{b}	218.6 ± 48.1^{d}	358,3 ± 133.0^{bc}	96,3 ± 36.4^{e}	177.0 ± 71.8^{de}
	Adhesive force (g)	-5.1 ± 1.1^{a}	-5,1 ± 1.0^{a}	-6,5 ± 1.0^{ab}	-5,7 ± 1.1^{a}	-5,8 ± 1.1^{a}	-5,9 ± 1.1^{a}	-6,7 ± 1.4^{ab}	-9,6 ± 1.9^{b}	-6,2 ± 0.9^{a}
Mechanical strength	Hardness (g)	394 ± 105.3^{ab}	453.6 ± 106.4^{a}	276.2 ± 82.5^{ab}	457.6 ± 166.6^{a}	386.9 ± 124.8^{ab}	79.3 ± 24.5^{c}	215.4 ± 83.9^{b}	26.5 ± 10.0^{d}	302 ± 78.1^{ab}
	Area cycle (gs)	2642.9 ± 516.8^{a}	3636.0 ± 798.2^{a}	1687.3 ± 502.9^{ab}	3295.7± 1023.3^{a}	2770.5 ± 1128.3^{a}	415.4 ± 75.4^{c}	1166.4 ± 214.2^{bc}	117.5 ± 35.4^{c}	2072.8 ± 504.8^{a}
	Fracture force (g)	3.1 ± 1.2^{a}	1.8 ± 1.2^{a}	2,6 ± 1.1^{a}	2.0 ± 1.2^{a}	3.5 ± 1.3^{a}	5.0 ± 3.0^{a}	12.0 ± 9.4^{a}	3,3 ± 2.1^{a}	3.8 ± 1.3^{a}
	Adhesive force (g)	-.8.1 ± 2.5^{a}	-7.1 ± 1.0^{a}	-7.1 ± 0.8^{a}	-7,2 ± 1.3^{a}	-6.2 ± 1.3^{a}	-10,1 ± 3.1^{a}	-15,3 ± 7.5^{a}	-9,9 ± 2.3^{a}	-7,7 ± 1.1^{a}

Hierarchical cluster analysis was also performed on the measured values of textural parameters above using the function of squared Euclidean distance in order to sort and allocate each variation of gel capsules into different homogenous groups (clusters), from then on, to clearly see the different levels of firmness and assess similarities or differences between each capsule variation on the basis of overall firmness (or softness). All the resultant clusters are depicted by a dendrogram seen in Figure 11. The cluster analysis clearly defined two greatly distinct groups at squared Euclidean distance of 25 (i.e. at

the highest possible dissimilarity level), one of which is related to harder, while the other one belongs to the softer capsule categories. Considering that (for example) gellan gum-xanthan gum capsules were clearly observed as soft capsules based on the measured values of each textural parameters (Table 7), the group encompassing the solely alginate and largely all the alginate-based capsules blended with prebiotic components (except the lactosucrose LS40L-alginate ones) obviously represented the harder category of the capsules, whereas the group comprised of chitosan coated alginate, gellan gum-xanthan gum, lactosucrose LS40L-alginae, κ-carrageenan-locust bean gum and DEAE Sephadex coated alginate capsules represented the softer types of the capsules. These two main groups could be further divided into five subgroups if dendrogram is cut at a shorter distance of 3.5 (i.e. at a lower dissimilarity level). Based on this level of clusterisation, we can see that lactosucrose LS55L-alginate and resistant starch-alginate capsules were more similar to each other than were to lactulose-alginate or alginate capsules on the aspect of firmness. However, especially the pair of chitosan coated alginate and gellan gum-xanthan gum capsules appeared to differ the most from the pair of lactosucrose LS55L-alginate and resistant starch-alginate capsules considering that these two clusters were generated farthest away from each other (having 'the furthest neighbouring' relationship) (Figure 11).

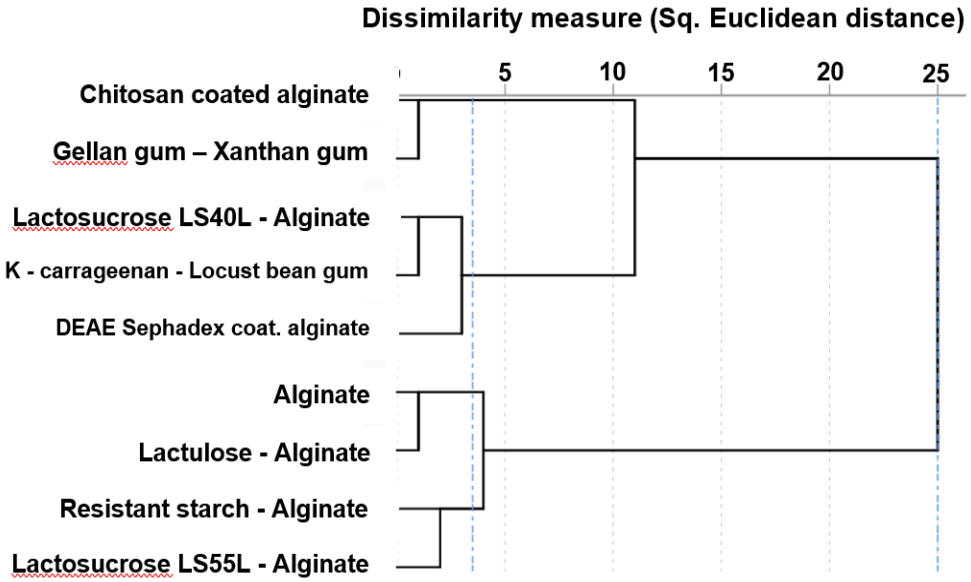


Figure 11. Dendrogram with a classification of different types of extrusion-derived gel capsules based on their overall textural attributes. Groups were formed according to squared Euclidean distances

In overall, these textural results indicated that the general mechanical characteristics of alginate capsules could be enhanced, and their physical stability could be improved especially by being reinforced with resistant starch. In addition, lactosucrose and lactulose were also found largely promising in this aspect. However, from the sensorial point of view, a soft texture that gellan gum-xanthan gum capsules had may be more favourable for mitigating somewhat the gritty or lumpy mouthfeel of these capsules when consumed with a food. The chitosan coating appeared to affect drastically the mechanical characteristics of alginate capsules, with giving them a much softer and looser gel matrix.

4.2.6 Viability of extrusion-based encapsulated and free *L. casei* 01 under simulated gastric and intestinal conditions based on a simple protocol

After gaining more information on the texture qualities (mechanical strength) of each gel matrix, free (unencapsulated control) and encapsulated *L. casei* 01 in these various gel formulations were subjected to sequential simulated gastric (SGF) and intestinal fluids (SIF) ; viability changes in these conditions were observed as a function of treatment time. This survival test was performed according to the protocol described by Krasaekoopt et al., (2004), with slight modifications. The gastric treatment of each capsule variation was run for four different durations of 0, 45, 90 and 135 min, while the subsequent intestinal treatment was run for 150 min. Furthermore, the impact of using 0.3 % (w/v) pepsin as an additional component in SGF was also explored in this experiment.

Figure 12. compares the cell survival profiles during the ***simulated gastric treatments*** with (A) and without (B) the pepsin involvement. It was clearly noticeable that the viability of the encapsulated *L. casei* 01 in SGF varied with different gel matrices of the capsules. Bacteria especially encapsulated in resistant starch-alginate, lactosucrose LS40L-alginate, lactosucrose LS55L-alginate and chitosan coated alginate capsules manifested better degree of gastric tolerance even in the presence of pepsin, as compared to free cells and alginate beads. In their cases, viable cells of more than 5.5 log CFU/g (60 %) could be protected after exposed to SGF for 45 min. Resistant starch-alginate, lactosucrose LS40L-alginate and lactosucrose LS55L-alginate capsules showed particularly high bacterial protection with viable cell counts averagely remained 6.0 log CFU/g (80 %), 4.0 log CFU/g (60 %) and 3.0 log CFU/g (40 %) after 45, 90 and 135 min, respectively. Lactulose-alginate capsules also showed promising protection ability, but only in the case of no pepsin contact, where viable cells of 6.83 log CFU/g (80 %), 4.41 log CFU/g (60 %) and 2.17 log CFU/g (40 %) were counted after treated for 45, 90 and 135 minutes, respectively. However, encapsulating into gellan gum-xanthan gum,

DEAE Sephadex coated alginate, and κ-carrageenan-locust bean gum did not improve the viability of cells against the simulated gastric conditions, compared to alginate beads. Similar survival outcome for gellan gum-xanthan gum and κ-carrageenan-locust bean gum capsules was also obtained with *L. reuteri* (Muthukumarasamy et al., 2006).

The presence of pepsin in the SGF was found to be critical during the gastric phase as better protection profile of the capsules was mostly obtained with the presence of pepsin in SGF; in more specific, higher bacterial viability was observed in the cases of alginate-, lactosucrose LS40L-alginate-, lactosucrose LS55L-alginate-, chitosan coated alginate, DEAE Sephadex coated alginate, and gellan gum-xanthan gum -based encapsulations. This result is in accordance with that reported by Liserre et al. (2007) for encapsulated *B. animalis* in several modified alginate matrices. However, it was contradictory when Zou et al. (2012) obtained that whey protein-based capsules did not protected well *B. bifidum* F-35 in SGF with pepsin activity. No clear effect of pepsin activity on the bacterial viability was also stated by Nag (2011) in the case of *L. casei* 431-loaded protein-polysaccharide gel capsules. Here, the better survival rate observed with the presence of pepsin may be partly attributed to the possibility of pepsin binding in some way to anti-gelling free Na^{+}-ions, otherwise the absence of this enzyme allows this anti-gelling cations to freely decompose the Ca-alginate gel structure by replacing Ca^{2+}-ions with themselves – even at this low pH (Chen & Chen, 2007; Clement et al., 1971; Cook et al., 2011). This can be also supported by that the κ-carrageenan-locust bean gum capsules with cross-linked by K+-ions was not significantly affected by the absence of pepsin effect. Further experimentation, however, is still needed to confirm it or to find out the actual explanation of this effect.

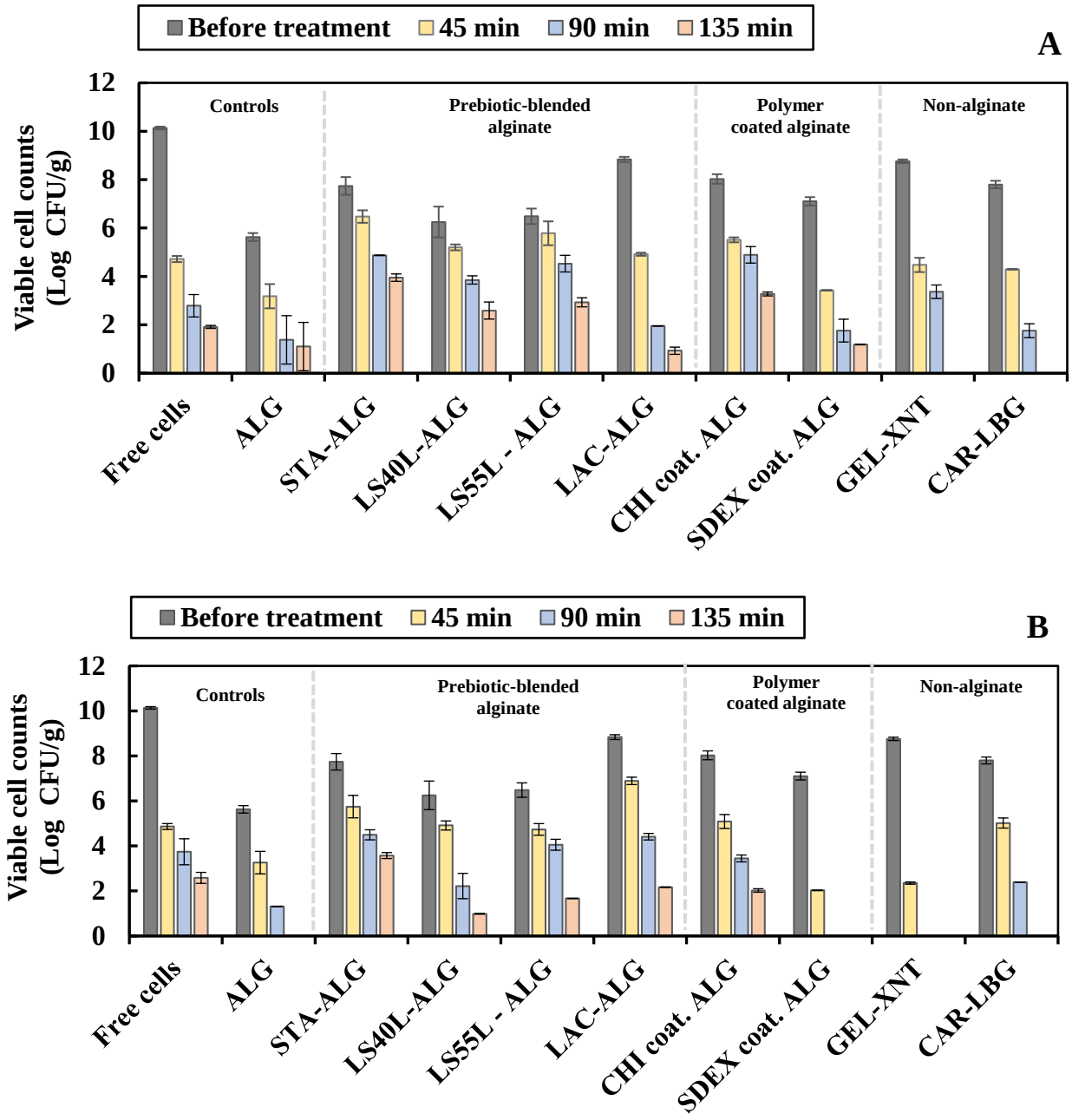


Figure 12. Survival profile of free and encapsulated *L. casei 01* cells in each variation of extrusion-formed gel capsules during the digestion process - with pepsin (A) and without pepsin (B) in simulated gastric fluid. Abbreviations: ALG, alginate; STA-ALG, resistant starch-alginate; LS40L - ALG, lactosucrose LS40L - alginate; LS55L - ALG, lactosucrose LS55L - alginate; LAC-ALG, lactulose - alginate; CHI coat. ALG; chitosan coated alginate; SDEX coat. ALG, DEAE Sephadex coated alginate; GEL - XNT, gellan gum – xanthan gum; CAR - LBG, κ-carrageenan – locust bean gum capsules

A subsequent ***bile salt-contained intestinal treatment*** was carried out right after the end of gastric treatment. Encapsulated bacteria especially in lactulose-alginate, resistant starch-alginate and lactosucrose LS55L-alginate capsules exhibited enhanced tolerance towards these intestinal conditions as compared to free cells (Figure 13). It is worth to note that alginate and lactosucrose LS40L-alginate beads failed to protect the bacteria during the passage through the bile salt conditions when they were previously pre-treated in the gastric conditions with the presence of the pepsin activity. Generally, the bile salts have potential toxic effect on viability of cells by disruption of cell membrane from lipid solubilisation and by acidification of cells after the entrance into the cytoplasm (Hay & Zhu, 2016; Kurdi et al., 2006).

Overall, the most effective protection could clearly be noted for resistant starch-reinforced beads, with which viable cells could be still detected even after 135 min of gastric and 150 min of intestinal treatment. Furthermore, by the end of simulated gastric treatment, live cell counts in this bead type reduced by only 3.79 log CFU/g (32%) and 4.17 log CFU/g (41%) with pepsin and without the presence of pepsin, respectively (Figure 12). This may be explained by the presence of resistant starch, promoting the prebiotic effect and/or decreasing the pore size of alginate gel matrix. Among the prebiotic included capsules, lactosucrose LS55L-alginate and lactulose-alginate are also promising ones in the probiotic-protection ability against the entire simulated gastric and intestinal conditions.

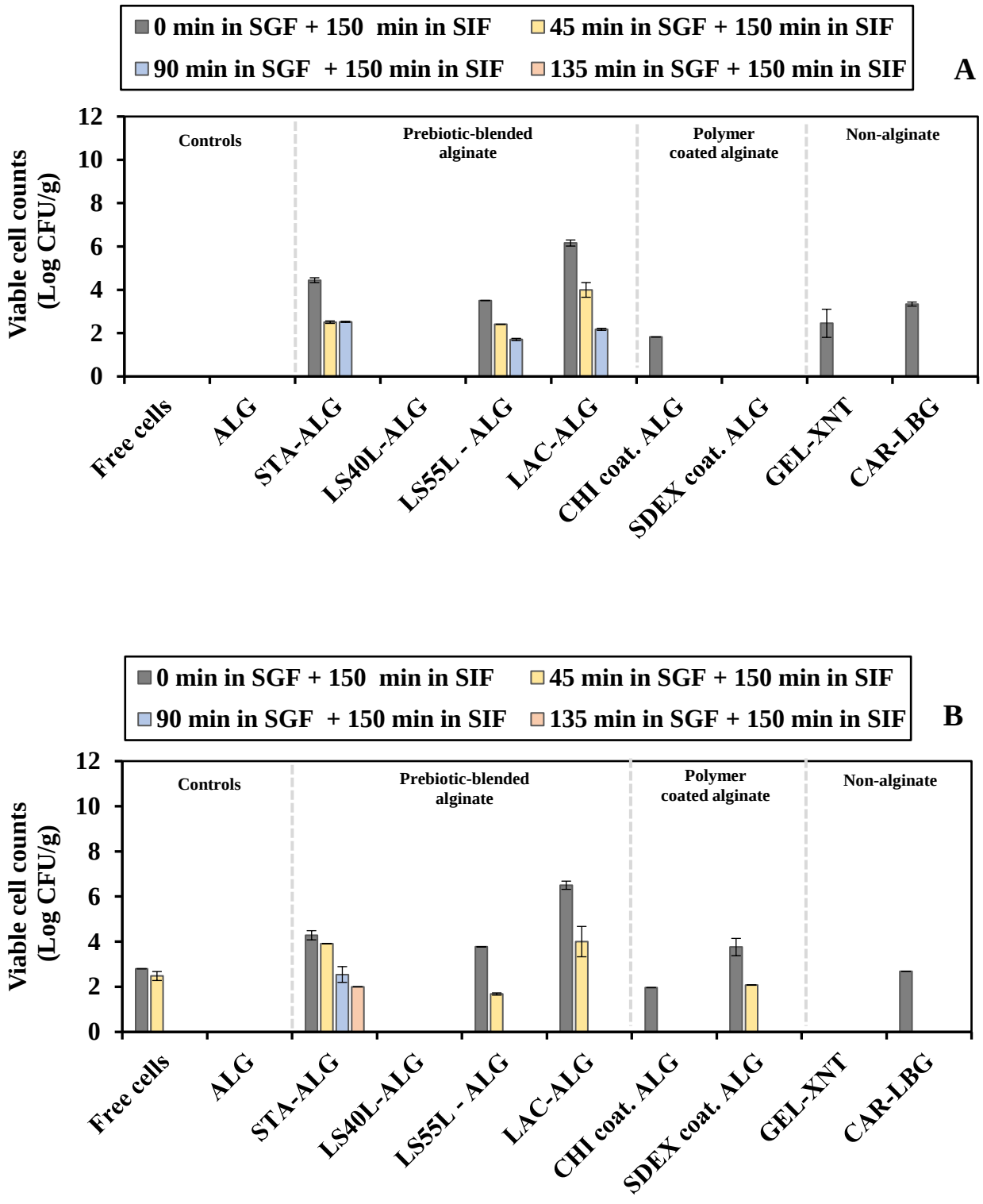


Figure 13. Survival profile of free and encapsulated *L. casei 01* bacteria in each variation of extursion-formed gel capsules during the sequential treatment in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). Graph 'A': with prior pepsin contact during the gastric phase; Graph 'B': without prior pepsin contact during the gastric phase

Several other studies (Ashwar et al., 2018; Iyer & Kailasapathy, 2005; Li et al., 2011; Martin et al., 2013) similarly demonstrated that encapsulation in starch-reinforced alginate gel beads increased the survival of probiotic cells treated in both highly acidic and bile salt environment, compared to those encapsulated in alginate and the free cells. Resistant starch-alginate capsules were found to be effective in gastric protection of *L. reuteri* PTA 4965 even at pH = 1.5 (Muthukumarasamy et al., 2006). However, in the study of Sultana et al. (2000), both resistant starch-alginate and alginate beads were not found to protect significantly better the probiotic *L. acidophilus* and *B. infantis* when subjected to *in vitro* gastrointestinal conditions. My present results related to the chitosan coated alginate capsules contradict that reported by Yeung et al. (2016), but agreed with the one by Krasaekoopt et al. (2004). Although formed with emulsion technique, Zou et al. (2011) found that alginate microspheres coated with chitosan provided better probiotic protection against *in vitro* gastrointestinal fluids than in the one reinforced with starch.

It was also experienced that the extent of bacterial protection under gastrointestinal conditions was not fully influenced by the textural characteristics of the gel capsules (see Section 4.2.5). For example, chitosan coated alginate and κ-carrageenan-locust bean gum capsules did not protect bacteria worse than the alginate capsules with firmer gel matrix. Although, gellan gum-xanthan gum capsules, with having the softest gel texture, showed one of the poorest bacterial protection.

4.2.7 Viability of extrusion-based encapsulated and free *L. casei* 01 with simulated digestion based on Infogest procedure

Besides the previous survival test in simulated gastrointestinal fluids based on a more simple and common protocol (Krasaekoopt et al., 2004), the survival of *L. casei* 01 encapsulated into some types of the capsules was also investigated by using a much more sophisticated simulated digestion based on the standardised Infogest *in vitro* model (Egger et al., 2016; Minekus et al., 2014). The aim of this digestion study was to assess how bacterial survival can be different if they are exposed to more complex digestive enzyme activities, electrolyte components and an additional oral (salivary) phase and thus to closer mimic the *in vivo* conditions of human digestion process. On the other hand, this specific static digestion protocol has been developed by COST Infogest network (2014) to harmonise several previous *in vitro* protocols reported for simulating human digestion and thereby to better compare the related viability results (Egger et al., 2016). The present digestion experiment was conducted with the blend of resistant starch-alginate- and the chitosan coated alginate-encapsulated cells as test samples, along with pure alginate-encapsulated and unencapsulated cells as control

samples. The former two capsule types were shortlisted for providing the most promising bacterial protection among either the blend or the polymer-coated types, during the *in vitro* digestion with simple protocol (Section 4.2.6). Furthermore, the reason behind this choice was also to compare the effect of blending and coating.

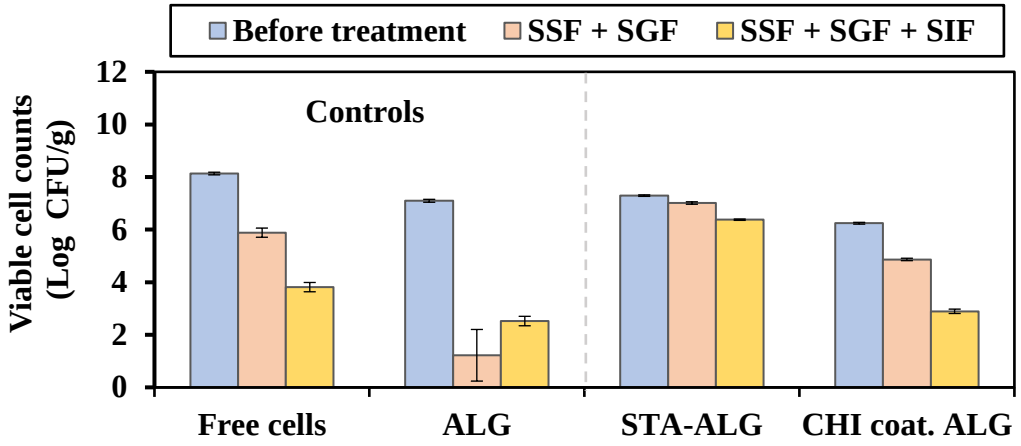


Figure 14. Viability of free and different extrusion-based encapsulated *L. casei 01* cells during the Infogest-based *in vitro* digestion. Viability counts were determined before the treatment, after the sequential treatments in simulated salivary fluid (SSF) (2 min) and simulated gastric fluid (SGF) (120 min), and after the following treatment in simulated intestinal fluid (SIF) (120 min). Abbreviations: ALG, alginate; STA-ALG, resistant starch – alginate; CHI coat. ALG, chitosan coated alginate capsules entrapping *L. casei* 01

The cell survival profiles during this digestion process are shown in Figure 14. These results again supported the great protection effect of resistant starch even in more complex digestion conditions, with a maximal loss of 1 log CFU/g over the whole digestion period. More specifically, the decrease in viable count was found this time to be not more than 0.28 log CFU/g after the salivary (SSF) and gastric (SGF) phases, and 0.63 log CFU/g after the subsequent intestinal (SIF) treatment; these losses are very minimal compared to those ones obtained with the simple gastrointestinal model (see Section 4.2.6). It is also worth to note that this starch-alginate-based encapsulation could maintain the number of survived bacteria cells (even from the initial viable number of 7.29 log CFU/g) above the recommended minimal viability level (> 6 log CFU/g or mL) necessary for the realisation of probiotic effects. On the other hand, the greatest loss of 2.25 log CFU/g, then 2.07 log CFU/g were seen with free cells. Surprisingly, the viability of encapsulated cells in chitosan coated alginate capsules did not differ significantly ($p > 0.05$) from that of free cells throughout the whole digestion, unlike what was

observed with the simple digestion protocol. With this sophisticated digestion protocol, every type of examined gel capsules was completely dissolved by the end of the whole digestion (after the intestinal phase).

In other similar studies, the viability of electrospraying - and freeze-drying-based encapsulated *L. plantarum* CECT 748 T cells were also tested with this digestion protocol (with slight modifications), after which they showed an average loss of 1.85 log CFU/g after the gastric phase, and an average loss of only 0.5 log CFU/g after the intestinal phase (Gomez-Mascaraque et al., 2016). Eratte et al. (2017) found that the viability of their encapsulated and co-encapsulated *L. casei* 431 cells decreased by 4.8 and 1.5 log CFU/ml, respectively, after 2 h of exposure of the roughly same simulated gastric fluid as in this study.

4.2.8 Thermal stability of extrusion-based encapsulated and free *L. casei* 01 under high-temperature conditions

In this study, the effect of the high thermal exposures typical of pasteurisation processes was additionally assessed on resistant starch-alginate encapsulated *L. casei* 01 cells. This capsule variation was evaluated owing to its outstanding bacterial protection exhibited during the previous survival tests in simulated digestion conditions (Section 4.2.6 and 4.2.7). For comparison, free and pure alginate encapsulated *L. casei* 01 bacteria were additionally involved as encapsulated (without resistant starch-blending) and unencapsulated controls. To this end, they were left immersed in a water bath set to either 60°C or 85°C, and their cell viability was determined after different treatment times of 2, 5, 10 and 20 min. Figure 15 presents these results for the heat-treatment at 60°C. The initial cell count prior to heat treatment were 10.11, 7.99 and 8.07 log CFU/g for free, alginate- and resistant starch-encapsulated probiotics, respectively.

L. casei 01 bacteria were found to be more resistant to 60°C in both encapsulated forms, especially in resistant starch-alginate matrix. More specifically, resistant starch-alginate capsules maintained some of the viability even after the 5-min treatment with the total loss of 3.37 log CFU/g, as opposed to the free and the solely alginate-encapsulated bacteria. However, bacteria could not survive even with resistant starch-alginate when the incubation was more than 10 min long. The protection ability of these capsules was much weaker when exposed to the higher temperature of 85°C, in a way that no viable cells could be detected even after 2-min treatment, initiating from similar viable counts (8-10 log CFU/g).

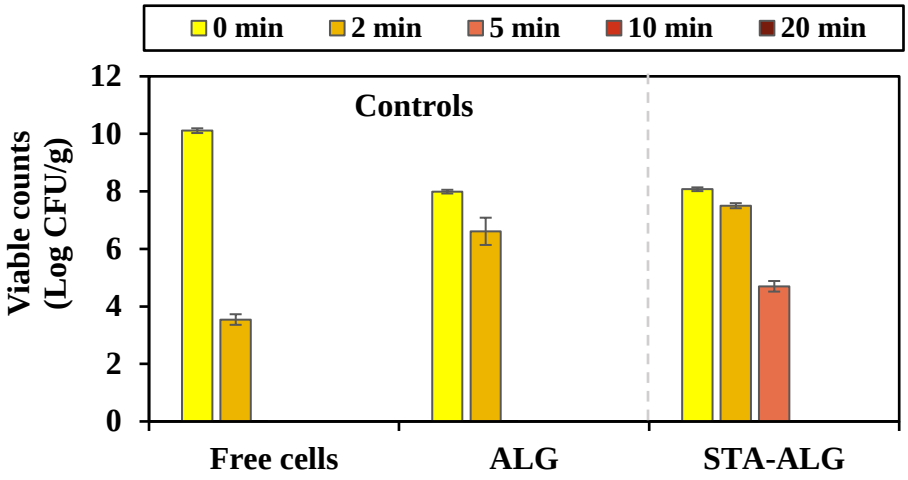


Figure 15. Viable bacterial count of free and extrusion-based encapsulated *L. casei* 01 in alginate (ALG) and resistant starch-alginate (STA-ALG) gel capsules after heat treatment at 60°C for 2, 5, 10 and 20 min

Mahmoud et al. (2020) observed a viability reductions of 1.41 log CFU/g, 3.06 log CFU/g, 5.03 log CFU/g and 2.82 log CFU/g for *L. plantarum* encapsulated into capsules composed of alginate-skim milk, alginate-chitosan, alginate-denatured whey protein and alginate-dextrin, respectively, after 30 min of heat-treatment at 65°C. Furthermore, resistant starch-alginate capsules provided better cell protection for *L. acidophilus* LA-5 in the study of Teoh et al. (2011), detecting a viability loss of only 1.99 logs after 30 min of heat exposure at 60°C.

4.2.9 Storage stability of extrusion-formed encapsulated and free *L. casei* 01 bacteria in different non-dairy food matrices at different temperatures

According to several previous studies, *L. acidophilus* bacteria tend to show less resilience than other species when applied in plant-based foods such as oat-based beverages or fruit drinks (Champagne & Gardner, 2008; Gawkowski & Chikindas, 2013; Gokavi et al., 2006; Lankaputhra & Shah, 1995; Rius et al., 1994). Based on this motive, long-term storage experiments were carried out with resistant starch-alginate encapsulated *L. casei* 01 bacteria in oat and beetroot drinks in order to evaluate the effect of encapsulation on the storage stability of bacteria. As controls, pure alginate encapsulated (without starch-blending) and free *L. casei* 01 bacteria were also evaluated in this storage experiment. The reason for choosing these different food matrices was to compare the effect of greatly different plant (i.e., cereal and vegetable-based) matrices on the viability of bacteria and the stability of the capsules. The storage was conducted both at 4°C and 20°C, for 5 months.

The applied drinks were fermented (37°C, 24 h) separately with free and each encapsulated *L. casei* 01 right before the storage experiment. For simplicity, these fermented drinks will be referred later as merely ‘oat drink’ and ‘beetroot drink’. During the storage periods viable cells in capsules and in free form were enumerated on a monthly basis. Along with it, changes in physicochemical attributes such as pH, acid and carbohydrate content were also monitored to compare the (possible) post-acidification ability of free and each encapsulated *L. casei* 01 over the storage period. In the case of both storages in oat and beetroot drink, the initial viable counts of free, alginate and resistant starch-alginate encapsulated *L. casei* 01 before the storage period were around 8.30, 9.30, and 9.22 log CFU/g, respectively.

4.2.9.1 Viability changes of encapsulated and free L. casei 01 in oat drink

By keeping at 4°C (Figure 16A), very minimal changes in viable cell counts were found both for alginate, resistant starch-alginate capsules and even for free cells over the whole storage periods. With this way, the recommended minimal level of 6 log CFU/g – for assuring probiotic effects – was maintained even after the 5-month storage. On the contrary, considerable decreases in viability were rather noted with the storage at 20°C (Figure 16B). These data revealed that encapsulation improved the long-term (5-month) storage stability of *L. casei* 01 in oat drink, especially with resistant starch-alginate. More specifically, the viability of free cells was undetectable after 4-month storage, whereas viable cells in resistant starch-alginate capsules were undetectable only after 5-month storage. Furthermore, reinforcing with resistant starch appeared to positively affect the protection ability of the alginate capsules at 20°C. Within this blend capsule matrix, the stability of *L. casei* 01 was extended by an additional month, and the total loss in their viability was determined to be around 4.3 log CFU/g after the 4-month storage (Figure 16B). It may be attributed to the presence of resistant starch in the alginate gel matrix which could exert a prebiotic effect on the bacteria and/or decrease the pore size of alginate gel matrix. However, in the case of short-term storage (up to 2 months), the stability of bacteria was not affected much by encapsulation, neither with resistant starch-alginate nor alginate.

Gokavi et al. (2006) experienced that the viability of free *L. acidophilus* only survived for 4 weeks of storage in oat beverage at 4°C. Furthermore, for my encapsulated *L. casei* 01, the viability loss of around 0.4 logs was observed after 1-month storage in oat beverage at 4°C. Conversely, in the case of same refrigerated storage in a yogurt dairy product, Krasaekoopt and Watcharapoka (2014) showed a greater viability loss of 1.6 log CFU/mL for *L. casei* 01 encapsulated in chitosan coated alginate

capsules (formed with extrusion) even by the end of 4-week storage. In the case of their equally encapsulated *L. acidophilus* LA-5, the viability loss was 2.7 log CFU/mL during this same period. When galactooligosaccharides were included in the alginate matrix, these viabilities in a yogurt product could be improved. Afzaal et al. (2019), with cold storing (4°C) alginate-encapsulated *L. acidophilus* in yogurt, also observed a greater final viable loss of around 1.5 logs than in my study even after 2 weeks of storage. They were also applied extrusion method for encapsulating their bacteria.

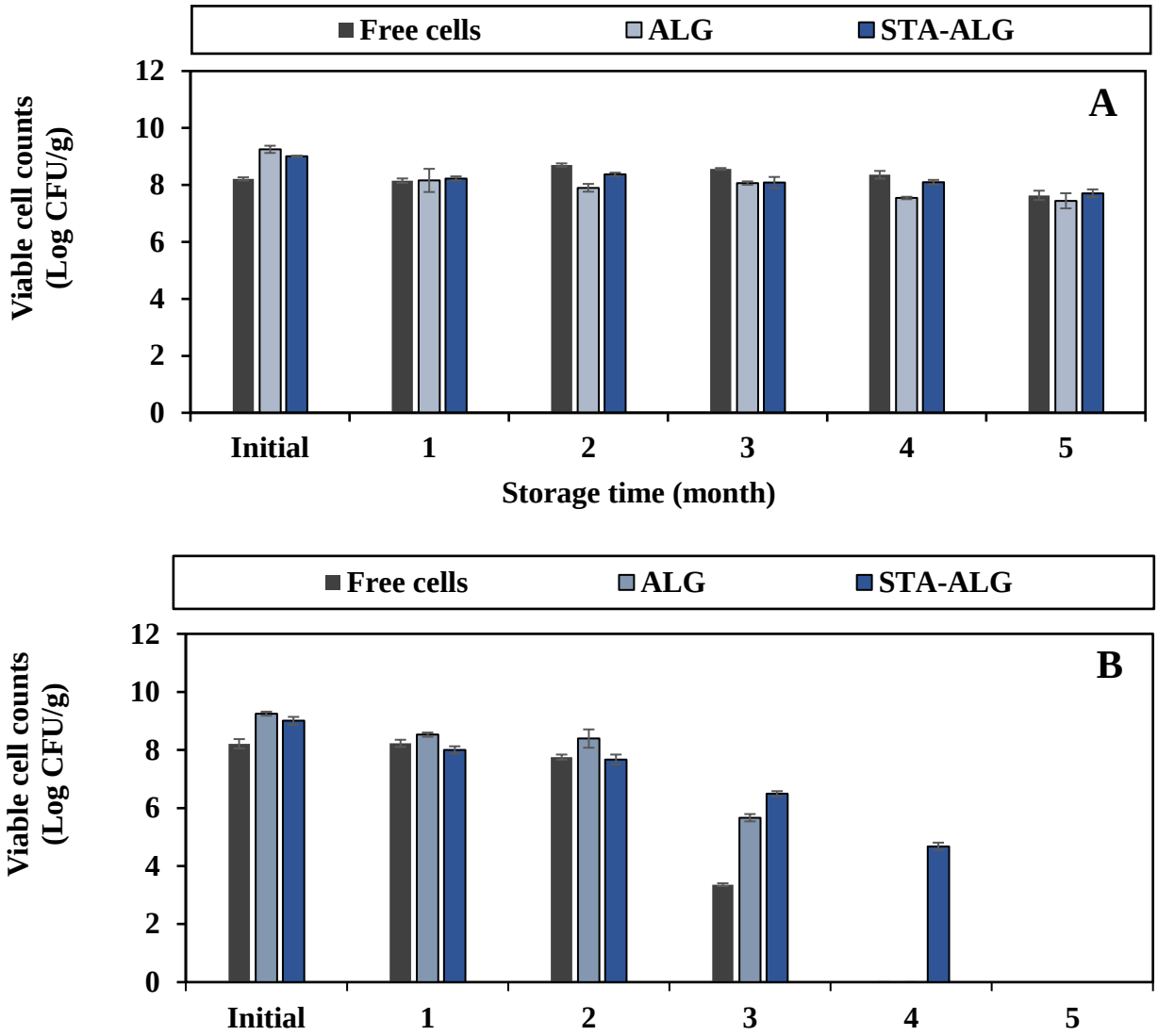


Figure 16. Viability of free and extrusion encapsulated *L. casei* 01 over the 5-month storage in oat drink at 4°C (A) and 20°C (B). Abbreviations: ALG, alginate encapsulated bacteria; STA-ALG, resistant starch – alginate encapsulated bacteria. 'Initial' refers to the viable counts detected right before the storage experiment but after the fermentation step

4.2.9.2 Effect of encapsulated and free bacteria on the pH, acid and glucose content of oat drink

The pH value of the raw oat drink was 6.7, which decreased to average 3.5 after fermentation. After fermentation, acidifications during storage were generally found minimal both at 4°C and 20°C, and only up to 1 month (Figure 17A and Figure 17B). By the end of this first month, pH reduced averagely to 3.2 at 4°C and to 2.7 at 20°C. At both temperatures, the acidification rate was noticed a little bit higher with encapsulated cells (both in alginate and resistant starch-alginate) than with free cells. In addition, the greatest pH drop was observed as 1.03 for the oat drink containing resistant starch-alginate encapsulated cells, resulting in pH 3.33. Similar pH drops were also recorded in case of yogurt inoculated with free and encapsulated *L. acidophilus* after 28 days of storage at 4°C (Afzaal et al., 2019).

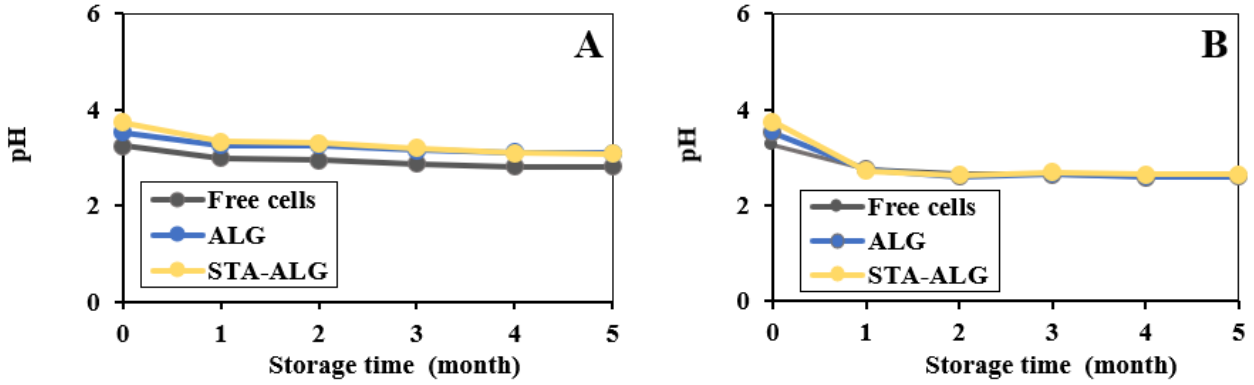


Figure 17. Effect of free and different extrusion encapsulated *L. casei* 01 on pH of oat drinks during 5-month storage at 4°C (A) and 20°C (B). Abbreviations: ALG, alginate encapsulated bacteria; STA-ALG, resistant starch – alginate encapsulated bacteria

To understand the acidification kinetics better, changes in acid and glucose content of each type of oat drink were also monitored throughout the storage; these results are represented in Figure 18 and Figure 19, respectively. These data reflect that there were indeed slight lactic acid productions in all types of the oat drink especially over the 1st month storage. After the 1st month, its concentration remained unchanged in all cases. Besides this acid component, a minimal degree of butyric acid production was also detected in oat drinks upon storage (Figure 18). It is worth to note that the degree of these acidification activities did not differ strikingly among free and each (alginate and resistant starch-alginate) encapsulated bacteria. By storing at 20°C, the lactic acid content increased by 0.16 w/v (%) to 0.31 % (w/v) with unencapsulated bacteria over the 1st month period, while its increasement rate with alginate and resistant starch-alginate encapsulated bacteria differed only by 0.07 – 0.11 w/v (%). Under the refrigerated conditions, overall changes in the lactic acid concentration

were even more negligible with an average increase of only 0.02 % (w/v), resulting average concentrations of 0.07 % (w/v) and 0.19 % (w/v) in encapsulated cell and free cell-contained drinks after the 1st month storage, respectively. The increasement rates of the butyric acid content among the different oat drinks containing different forms of bacteria were averagely 0.16 % (w/v) at 4°C – ending up at a concentration of around 0.38 % (w/v) – , and 0.11 % (w/v) at 20°C – ending up at a concentration of around 0.33 % (w/v) by the end of the whole storage experiment.

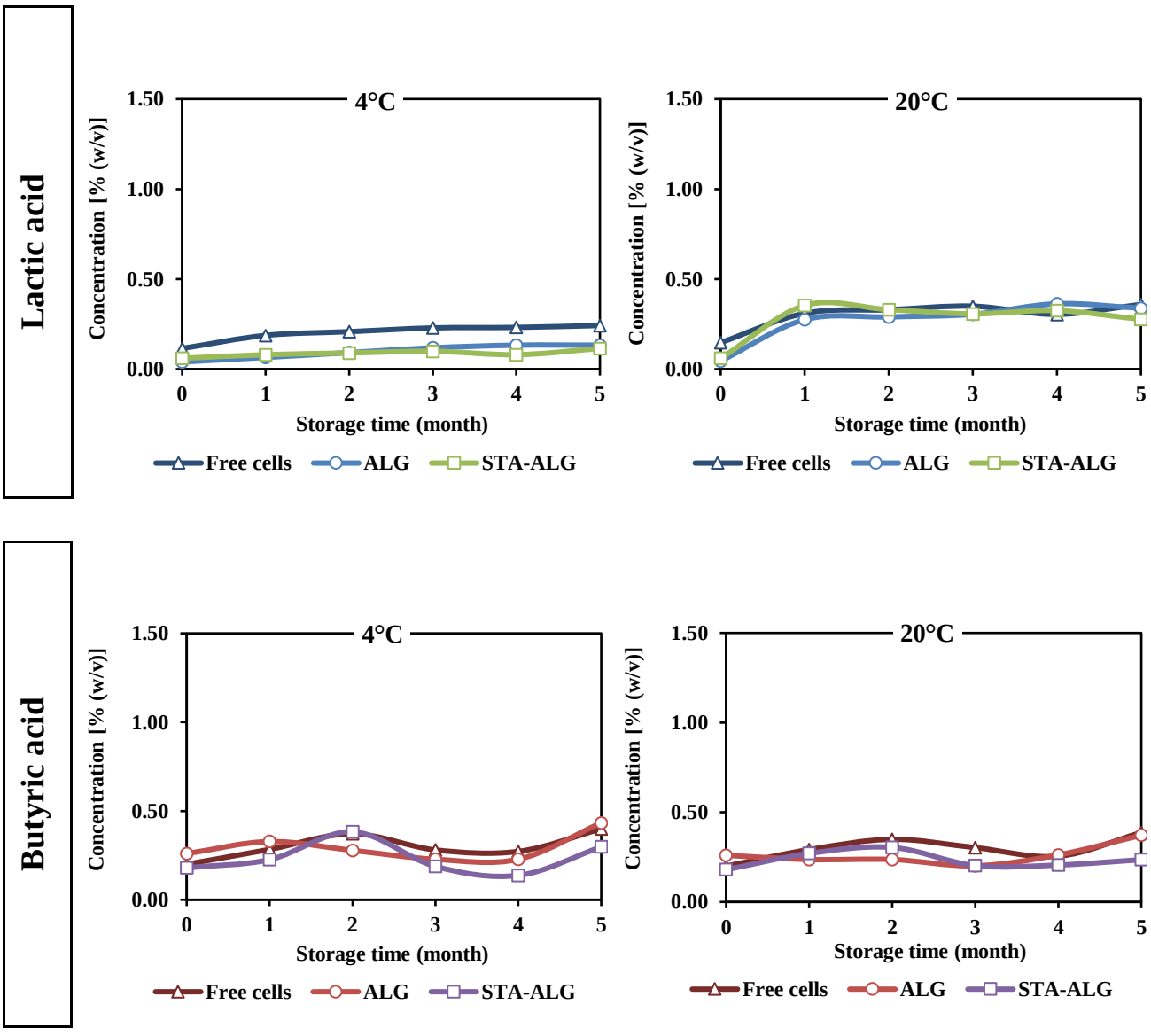


Figure 18. Effect of free and different extrusion-formed encapsulated *L. casei* 01 on the lactic and butyric acid content of oat drinks during storage at 4°C and 20°C. Abbreviations: ALG, alginate encapsulated bacteria; STA-ALG, resistant starch – alginate encapsulated bacteria

As was the case with the acid components, the change rates in the content of glucose and disaccharide did not vary considerably with (neither type of) the encapsulated bacteria, compared to the free cells. While lactic acid concentration increased over the 1st month storage at 20°C, glucose content showed notable decreasing trend by averagely 0.5 % (w/v) and ended up at a concentration of 0.24 % (w/v) especially during this same storage period (Figure 19); this then was followed by either level-off , ending up at around 0.09 % (w/v) with free and alginate encapsulated cells, or minor decrease to 0.02 % (w/v) with resistant starch-alginate encapsulated cells at the end of the storage. Under refrigerated conditions (4°C), glucose content largely decreased by only 0.14 (w/v) on average and ended up at a concentration of around 0.61 % (w/v) by the end of the whole (5-month) storage experiment. In fact, this glucose level was rather constant with alginate encapsulated bacteria at 4°C. As opposed to glucose, disaccharide content in oat drinks barely changed, remaining at a concentration around 1.0 % (w/v) throughout the whole storage at both 4°C and 20°C (Figure 19).

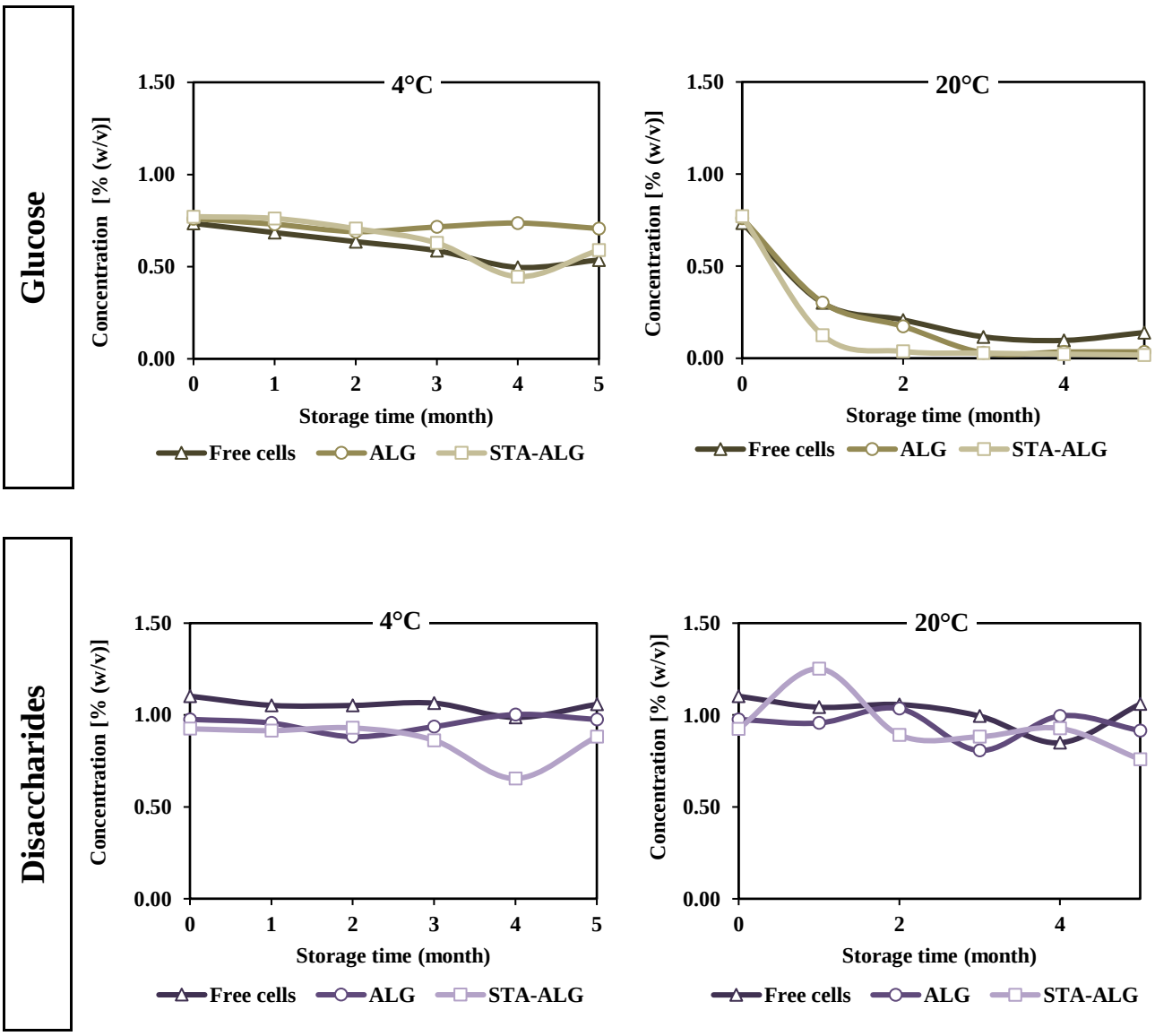


Figure 19. Effect of free and different extrusion-formed encapsulated *L. casei* 01 on glucose and disaccharide content of oat drinks during storage at 4°C and 20°C. Abbreviations: ALG, alginate encapsulated bacteria; STA-ALG, resistant starch – alginate encapsulated bacteria

4.2.9.3 Viability changes of encapsulated and free L. casei 01 *in beetroot drink*

By storing *L. casei* 01 in beetroot juice at 4°C (Figure 20A), a great deal of the viable cells could be protected with whichever types of encapsulation, with total losses of 2.2 log CFU/g and 2.7 log CFU/g in the case of alginate- and resistant starch-alginate-based encapsulation, respectively. With this way, viability level could be maintained averagely at 6.95 log CFU/g even after 5 months of storage, which fulfills the recommended minimum level (> 6 log CFU/g) needed for the realisation of probiotic effects. In contrast, the viability of bacteria reduced drastically by 5.7 log CFU/g over 5 months of

storage without any encapsulation, to the level of 2.75 log CFU/g. In accordance with my prior expectation, the stability was even less maintained at 20°C than it was at 4°C (Figure 20B), which was possibly caused by the higher tendency of bacterial acidification and pH reduction of the juice at such ambient temperature – as can be seen later in Figure 21 and in Figure 22. Free bacteria did not survive the 4-month storage, while encapsulated bacteria did not survive the 5 months of storage. Encapsulation with either alginate or resistant starch-alginate capsules resulted bacterial survival level of log 6.30 CFU/g by the 4th month storage at 20°C.

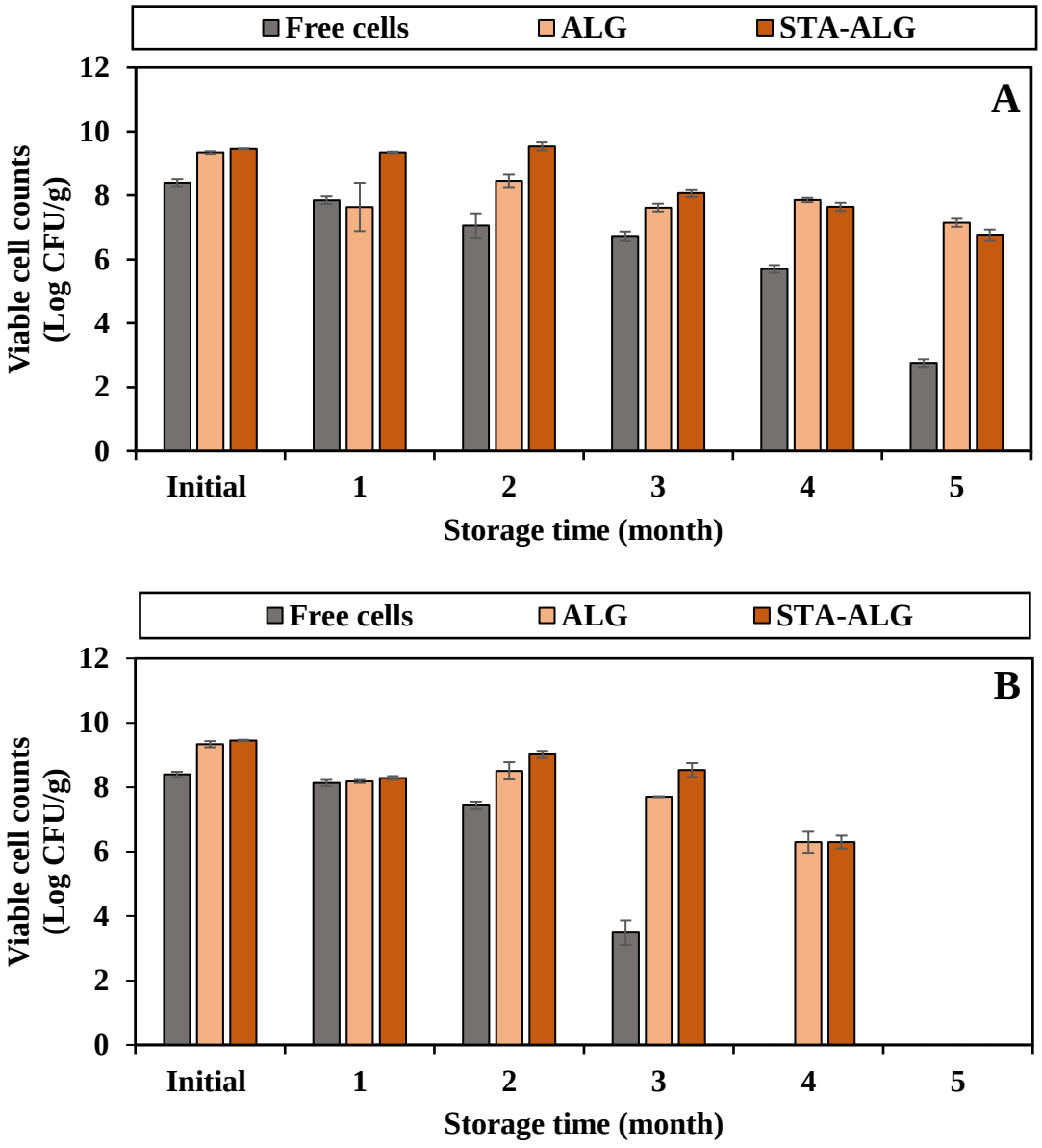


Figure 20. Viability of free and extrusion encapsulated *L. casei* 01 over the 5-month storage in beetroot drink at 4°C (A) and 20°C (B). Abbreviations: ALG, alginate encapsulated bacteria; STA-ALG, resistant starch – alginate encapsulated bacteria. 'Initial' refers to the viable counts detected right before the storage experiment but after the fermentation step

Overall, these results indicated that encapsulation did again positively affect the long-term storage stability of *L. casei* 01 in beetroot juice but only in the case of at least 3 months' storage time, like what was observed with the storage in oat drink (Section 4.2.9.1). However, the bacterial protection effect of encapsulation was seen not only at 20°C but also at 4°C. (Figure 20). Moreover, as opposed to the storage in oat drinks, blending alginate gel matrix with resistant starch did not exhibit much better protection for the bacteria in beetroot juice than without that.

Chaikham and Apichartsrangkoon (2012) did also evaluated the storage stability of alginate encapsulated *L. casei* 01 in a plant-based beverage, namely longan juice, with which they detected a viable cell decrease from 9 to 6 log CFU even after 4 weeks of storage at 4°C. On the contrary, a continuous increase in the viable bacteria of alginate-based encapsulated *L. casei* – prepared with a vibration technology – was observed by Olivares et al. (2019); in this case, they stored them in various pasteurised low-pH fruit juices, such as pineapple (pH 3.28), orange (pH 3.45) and raspberry juice (pH 2.75) for 28 days at 4°C. Cold storing in another low-pH fruit juice like pomegranate juice (pH 3.2), extrusion-formed alginate capsules failed to protect *L. plantarum* bacteria over the 4th week, thereby not differing from that in free form (Nualkaekul et al., 2012). Compared to the storage stability observed with beetroot drink in my study, Krasaekoopt and Watcharapoka (2014) reported a slightly greater viability loss for the same alginate encapsulated *L. casei* 01 after around 1 month of storage in orange juice at 4°C. Although, they also reported that incorporating galactooligosaccharides, as prebiotics, into the alginate gel matrix could improve the storage stability of this bacteria in orange juice.

4.2.9.4 Effect of encapsulated and free bacteria on the pH, acid and glucose content of beetroot drink

After the fermentation, the pH of raw beetroot drink fell to 4.2 from 6.0. During the subsequent storage, minimal reductions in pH were detected at both 4°C and 20°C storage temperatures (Figure 21). The greater pH reductions typically occurred only after the 1st month storage, regardless of the type of the beetroot drink. After this period, the pH reduced averagely to 3.9 under refrigerated conditions (4°C) and to 3.5 under ambient conditions (20°C). Considerable difference was not observed among the beetroot drink varieties as to the rate of pH decrease. These similar results were also found with the storage in oat drinks (Section 4.2.9.2).

According to the results obtained from HPLC analysis, it was found out that lactic acid content barely increased in beetroot drinks during the storage, steadily remaining at a concentration of around 0.33 % (w/v) with 4°C and around 0.38 % (w/v) with 20°C storage. Instead, more intensive production of butyric acid was rather observed especially at 20°C. Regardless of the types of beetroot drink, butyric acid increased averagely from 1.05 to 1.71 % (w/v) over the 1st month storage, then constantly decreased for the rest of the storage period to 1.13 % (w/v). With cold storage, this specific acid slightly increased from 1.14 to 1.29 % (w/v) with encapsulated cells and from 0.88 to 1.21 % (w/v) with free cells over 2 months period; this was then followed by constant decrease, ending up with a concentration of around 1.09 % (w/v) (Figure 22). The present of this specific acid was also manifested by butterish odour developed in each beetroot drink during the storage, which would deteriorate the sensory characteristics of beetroot drink. It is also worth to mention that the metabolic profile of these two acid components did not considerably differ whether bacteria were encapsulated or not, and also whether alginate or resistant starch-alginate gel matrix was used for encapsulation.

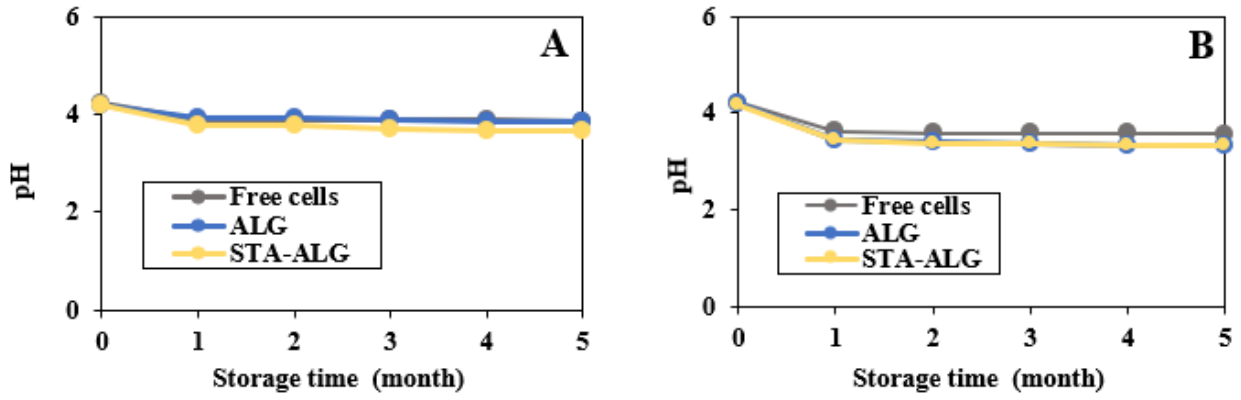


Figure 21. Effect of free and different extrusion encapsulated *L. casei* 01 on pH of beetroot drinks during 5-month storage at 4°C (A) and 20°C (B). Abbreviations: ALG, alginate encapsulated bacteria; STA-ALG, resistant starch – alginate encapsulated bacteria

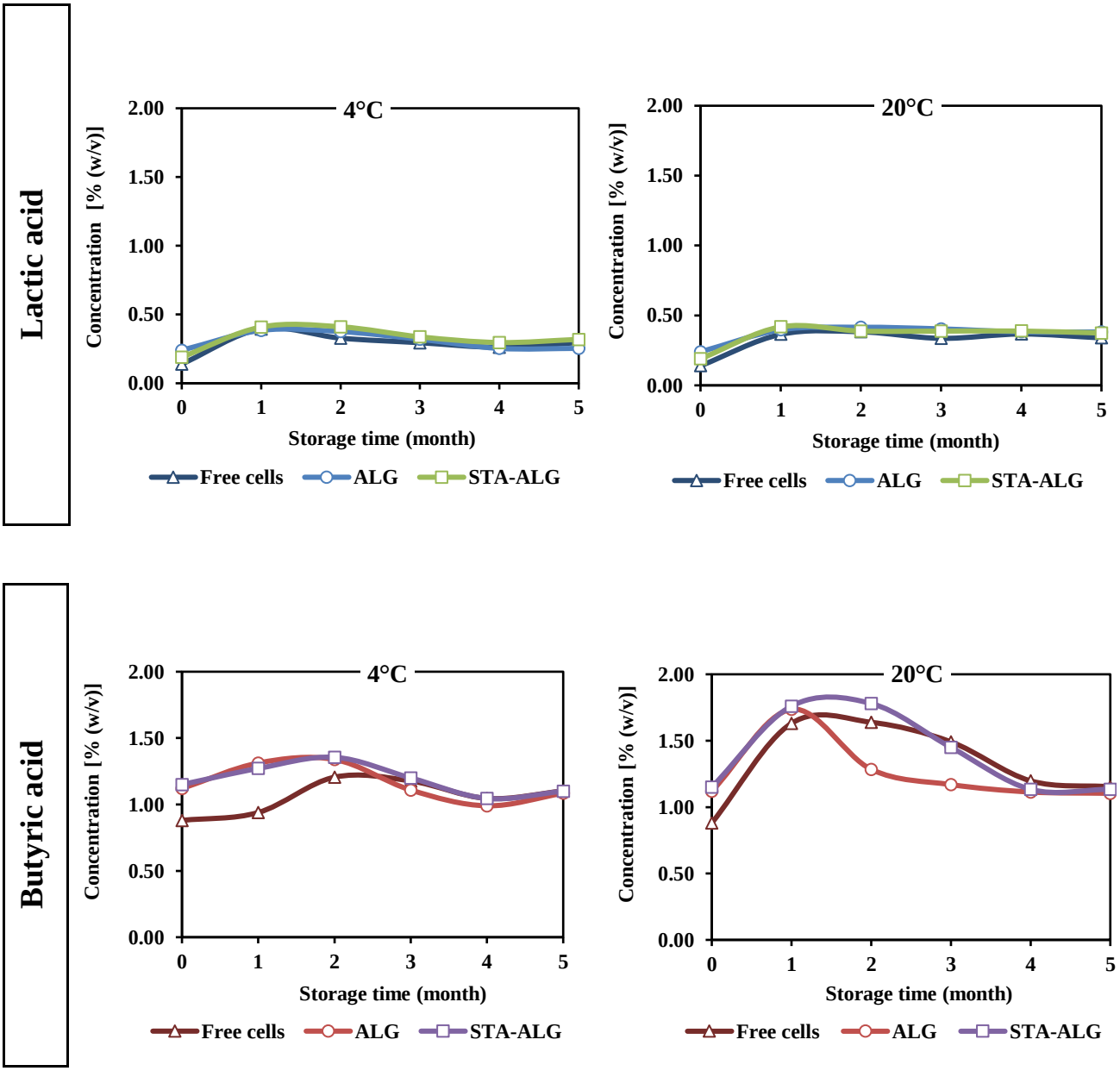


Figure 22. Effect of free and different extrusion-formed encapsulated *L. casei* 01 on the lactic and butyric acid content of beetroot drinks during storage at 4°C and 20°C. Abbreviations: ALG, alginate encapsulated bacteria; STA-ALG, resistant starch – alginate encapsulated bacteria

Comparing these results achieved for the oat-based drinks (Section 4.2.9.2), greater acid production was detected in beetroot drinks, which is possibly due to their higher content of simple sugar. These higher acidity levels determined for beetroot drinks – especially with storage at 20°C – could partially cause the decrease in the survival rate of free bacteria, which was eventually enhanced with

encapsulation (Figure 20). Also, the butyric acid production was generally minimal in oat drinks compared to beetroot drinks, regardless of the examined bacteria varieties (alginate-, resistant starch-alginate-encapsulated or free bacteria) and the storage conditions.

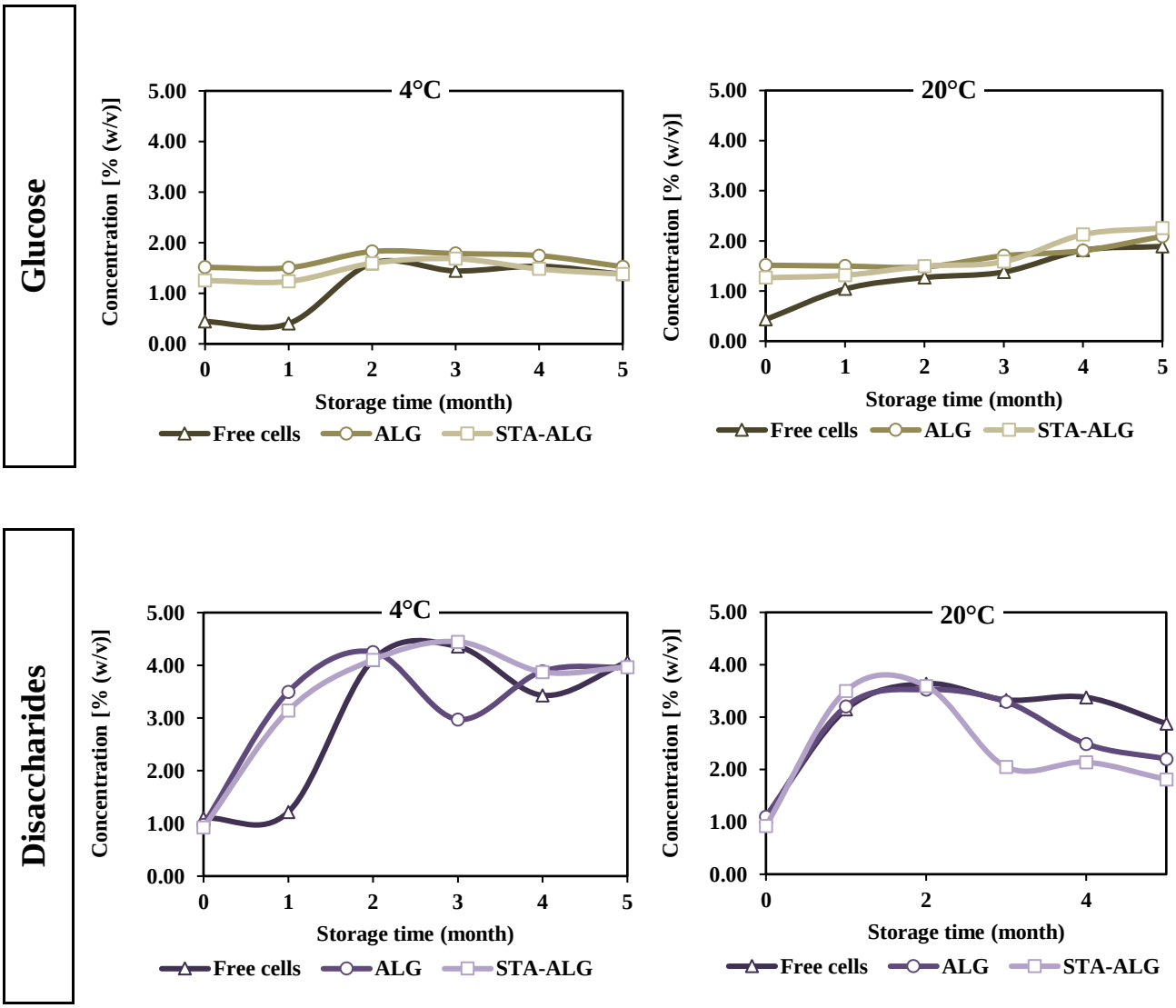


Figure 23. Effect of free and different extrusion-formed encapsulated *L. casei* 01 on glucose and disaccharide content of beetroot drinks during storage at 4°C and 20°C. Abbreviations: ALG, alginate encapsulated bacteria; STA-ALG, resistant starch – alginate encapsulated bacteria

Monitoring the simple sugar content in beetroot drinks, largely increasing trends were observed in the concentration of glucose and disaccharides at both 4°C and 20°C storage temperatures (Figure 23). Considerable difference in their change rates was again not observed among the free cell and the

two (alginate, resistant starch-alginate) encapsulated cell-contained varieties, using whichever storage temperature. In the cold stored (4°C) drinks with encapsulated cells (alginate, resistant starch-alginate), glucose content slightly increased averagely from 1.38 to 1.71 % (w/v) over the first 2 months of storage then decreased to the level of 1.45 % (w/v), whereas glucose content increased from 0.44 to 1.59 % (w/v) over the same storage period then decreased to the level of 1.38 % (w/v) in the free cell-contained drink. Also at 4 °C, disaccharide content averagely increased from 1.0 to 4.0 % (w/v) by the end of 5 month's storage. At 20 °C, glucose content steadily increased averagely from 1.0 to 2.0 % (w/v), while disaccharide content increased from 1.0 to 3.6 % (w/v) over 2 months then decreased to 2.3 % (w/v). These sugar accumulations were probably aroused from the degradation of oligosaccharide components in beetroot drinks upon storage, since decreasing concentrations in their content could be mostly detected with the HPLC analysis. More specifically, oligosaccharide content decreased averagely by 26 % with 4°C and by 54 % with 20°C storage. For this reason, it was impossible to acquire exact details about the actual utilisation of simple sugars merely based on these results. Increased sugar content upon storage (both at refrigerated and at room temperatures) was also reported in several studies for fruit and vegetable juices – without inoculated with any bacteria (Kausar et al., 2012; Mgaya-Kilima et al., 2014; Tabikha et al., 2010).

4.2.9.5 Other changes in stored oat and beetroot drinks

Apart from the previously presented factors, changes in capsule size and juice colour were also observed in the course of the storage. As shown in Table 8. with respect to the size of these capsules, significant capsule shrinkages were observed especially when stored in oat drink and at 20°C. Besides, resistant starch-alginate gel was generally observed as more instable than alginate gel in this regard, from which I inferred that the leakage of resistant starch into the external juice medium could happen during the storage, regardless of the storage temperature. Apart from this, size reductions could be general attributed to the same reason as reported by Sheu and Marshall (1993) – that is, alginate gel structure could be fortified additionally with calcium ions present in these drinks, by which the bound water in its gel was possibly removed. Further to this, it can be considered that commercial oat drink products is normally richer in calcium than the beetroot drink, based on the USDA Food Composition Database (2020). It should be noted that the size measurements were carried out based on the water-displacement method using a cylinder. This was necessarily done to avoid any inaccuracy caused by the possible dehydration and shrinkage of the capsules if left in the open air.

Table 8. Changes in diameter (mm) of extrusion-formed alginate (ALG) and resistant starch-alginate (STA-ALG) capsules after 5-month storage in different drinks. Values are presented as mean ± standard deviation based on 15 measurements

Capsule type	Before storage	After storage			
		Oat drink		Beetroot drink	
		4°C	20°C	4°C	20°C
ALG	2.78 ± 0.64	2.37 ± 0.23*	1.72 ± 0.31**	2.50 ± 0.19^{ns}	2.33 ± 0.31*
STA-ALG	3.19 ± 0.46	2.23 ± 0.26**	2.01 ± 0.04**	2.48 ± 0.23**	2.36 ± 0.32**

Significant differences as compared to the initial diameter of respective capsule type are indicated by * ($p < 0.05$), ** ($p < 0.001$), and non-significances are indicated by ns.

After about 3 months of storage at 20°C, there was a change noticed in the vivid colour of each examined types of beetroot drink, whereas the colour of the oat drinks did not change in any case (Figure 24). The colour alteration observed for the beetroot drinks could be effected by the oxidation of their pigment components like betalain (Akhavan & Jafari, 2017), which was likely accelerated with the storage at 20°C.

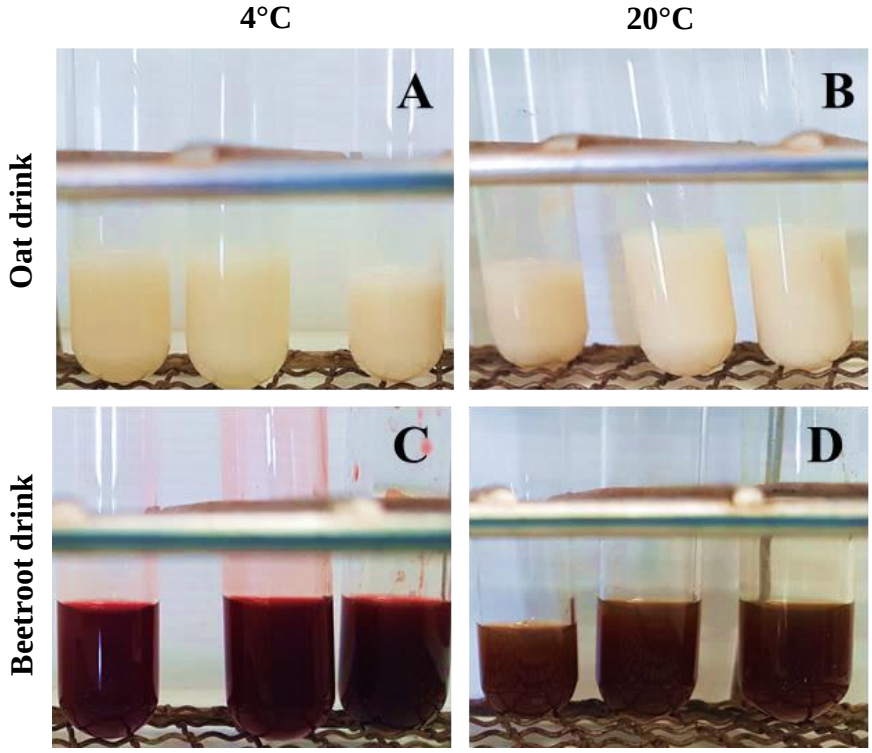


Figure 24. Photographs of oat drinks and beetroot drinks after 5 months of storage at 4°C and 20°C. The exact content of the three test tubes on each picture (from left to right): oat/beetroot drinks with unencapsulated, alginate encapsulated and resistant starch-alginate encapsulated *L. casei* 01

4.2.10 Preparation and encapsulation yield of extrusion-formed capsules containing *B. lactis* Bb-12

The extrusion-based encapsulation was also performed with another probiotic strain of *B. lactis* Bb-12 to find out how the same extrusion-formed capsules fare in entrapping and protecting a *Bifidobacterium* strain as compared to the case with the *Lactobacillus* one. In this study, only some of the gel capsule varieties, namely resistant starch-alginate, LS55L lactosucrose-alginate and lactulose-alginate capsules were chosen to be further studied for their promising performance in the protection of *L. casei* 01 during the survival test in simulated digestion fluids (see Section 4.2.6). The latest one was selected for its good protection ability against simulated intestinal fluid (SIF). Figure 25 shows the encapsulation yields obtained for *B. lactis* Bb-12. Extrusion-based technique resulted high *B. lactis* Bb-12 loading yields of 90 %, 89 % and 84% into resistant starch-alginate, lactosucrose LS55L-alginate, and lactulose-alginate capsules, respectively. Considering these results, no significant difference ($p > 0.05$) in encapsulating yield was found among the capsules formed from these specific materials. Also, higher encapsulation yields could be achieved for this bifidobacterial strain – especially with resistant starch-alginate and lactosucrose LS55L-alginate – than what obtained for *L. casei* 01. More specifically, encapsulation yields of *B. lactis* Bb-12 were higher by around 10%, in relation to those of *L. casei* 01 (Section 4.2.4).

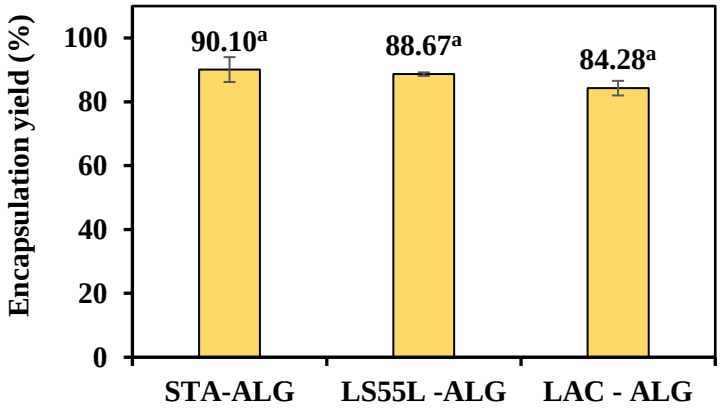


Figure 25. Encapsulation yield of extrusion-formed resistant starch – alginate, lactosucrose LS55L – alginate, and lactulose – alginate capsules containing *B. lactis* Bb-12. Same letter (a) next to the percentage values indicates non-significant differences ($p > 0.05$). Abbreviations: STA-ALG, resistant starch – alginate; LS55L-ALG, lactosucrose LS55L – alginate; LAC-ALG, lactulose – alginate-based encapsulation

4.2.11 Viability of extrusion-based encapsulated and free *B. lactis* Bb-12 under simulated gastric and intestinal conditions based on a simple protocol

The survival test with *in vitro* gastrointestinal fluids based on the simplified protocol (Krasaekoopt et al., 2004) was likewise conducted with encapsulated *B. lactis* Bb-12 within the above-mentioned capsule matrices (resistant starch-alginate, lactulose-alginate and lactosucrose LS55L-alginate) and free *B. lactis* Bb-12 as a control. In this case, gastric treatment of each sample was run for only two time-lengths (0 min and 135 min) and only with pepsin activity. However, the intestinal phase also lasted for 150 min as in the previous same survival tests (Section 4.2.6).

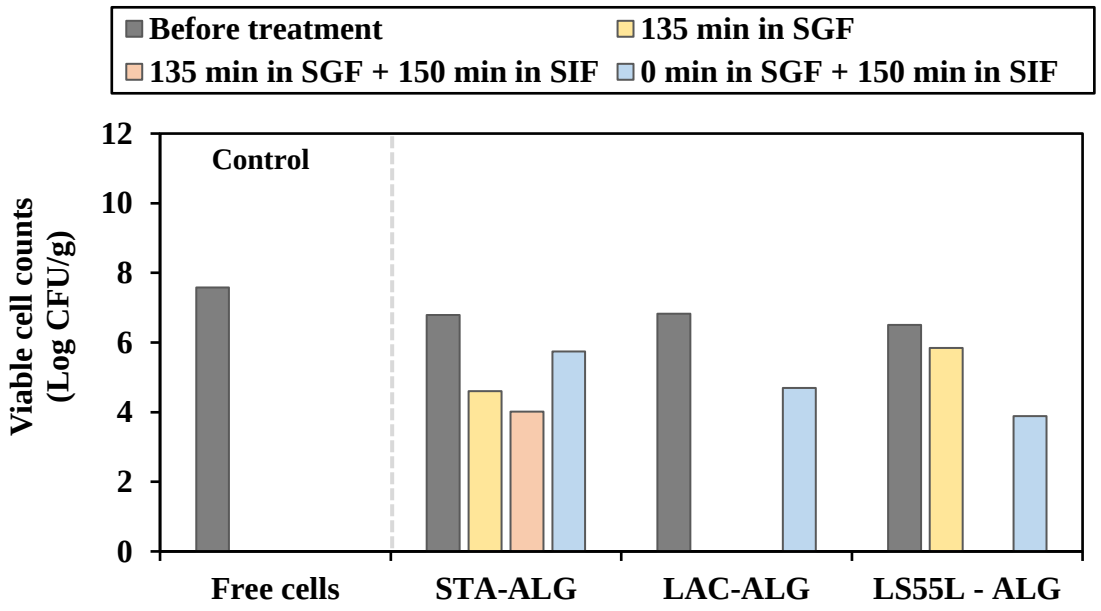


Figure 26. Survival profile of free and extrusion-based encapsulated *B. lactis* Bb-12 bacteria in each gel capsule type during the survival assay with simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). Other abbreviations: STA-ALG, resistant starch – alginate; LAC-ALG, lactulose – alginate; LS55L – ALG, lactosucrose LS55L – alginate capsules

The initial viable cell counts were 7.58 log CFU/g for the free cells, and around 6.70 log CFU/g for the encapsulated cells. As it can be seen from Figure 26, the free (control) cells exhibited the poorest survival throughout the whole survival assay, while the encapsulation generally provided an enhanced viability protection for *B. lactis* Bb-12. Although, these bacteria encapsulated with lactulose-alginate were likewise failed to remain viable when exposed to simulated gastric fluid (SGF) (including pepsin); compared to this result, *L. casei* 01 strain in lactulose-alginate capsules performed much better in the previous same gastric survival test (Section 4.2.6).

During the sequential incubation in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), the viable cell counts of *B. lactis* Bb-12 were best sustained when incorporated into resistant starch reinforced ALG; in this case, the total viability loss of only 2.77 log CFU/g occurred. Resistant starch-alginate matrix also provided the best protection for *L. casei* 01 (Section 4.2.6). However, *B. lactis* Bb-12 appeared to be a bit more resistant to the same simulated gastrointestinal conditions. More specifically, a greater viable cell loss of 3.79 log CFU/g was detected for *L. casei* 01 after the 135 min of SGF treatment and no viable count was found by the end of subsequent 150 min treatment in SIF.

As opposed to *B. lactis* Bb-12 encapsulated in resistant starch-alginate, no viable bacteria could be detected in lactosucrose LS55L-alginate capsules by the end of the sequential exposure to both digestion fluids. However, encapsulation with this type of capsule matrix resulted a much better gastric survival rate than that with resistant starch-alginate, losing viable cell counts by only 0.66 log CFU/g.

In addition, it was also experienced that encapsulated *B. lactis* Bb-12 showed higher tolerance when solely exposed to SIF (without the prior SGF treatment). Viewing from this aspect, resistant starch-alginate capsules can particularly be considered as excellent encapsulant for effectively maintaining the cell viability even with the prior treatment in SGF conditions. In the study of Sultana et al. (2000) encapsulation with both resistant starch-alginate and alginate were not reported to significantly improve the protection of *L. acidophilus* and *Bifidobacterium spp.* when subjected to *in vitro* gastrointestinal conditions.

4.2.12 Thermal stability of extrusion-based encapsulated and free *B. lactis* Bb-12 under high-temperature conditions

Following the survival test in simulated gastrointestinal fluids, heat tolerance was also assessed for resistant starch-alginate encapsulated and free (control) *B. lactis* Bb-12. This specific gel capsule type was ‘shortlisted’ for previously performing the best *B. lactis* Bb-12 protection against *in vitro* gastrointestinal conditions (Section 4.2.11). As with *L. casei* 01, bacteria were treated in a water bath at both 60°C and 85°C, during which their cell viability was determined after various treatment times. Figure 27 presents these heat tolerance results in the case of 60°C treatment. The initial viable cell counts prior to the heat exposure were 7.83 log CFU/g for the free cells, and 6.73 log CFU/g for the encapsulated cells.

Without encapsulation, *B. lactis* Bb-12 failed to survive even the 2-min treatment at 60°C, whereas encapsulation into resistant starch-alginate capsules drastically improved their heat tolerance in a way that around 60 % of the initial viable cells could be still detected after 20 min treatment. More

specifically, viable cells decreased by 2.28 log CFU/g, ending up at a level of 4.45 log CFU/g after the 20 min treatment. A striking different was also found when the heat protection effect of resistant starch-alginate-based encapsulation was compared between what obtained for *B. lactis* Bb-12 and *L. casei* 01 (Section 4.2.8) under the same 60°C conditions. While viable count of around 5.40 log CFU/g was still detected for the encapsulated *B. lactis* Bb-12 after 10 min of treatment, the encapsulated *L. casei* 01 bacteria did not survive this same degree of heat treatment. Interestingly, the bifidobacterial strain in unencapsulated form exhibited a bit poorer tolerance to this heat treatment than the free lactobacilli strain did. Although, it could be influenced by the lower initial viable cell counts (detected right before the treatment) of the bifidobacteria.

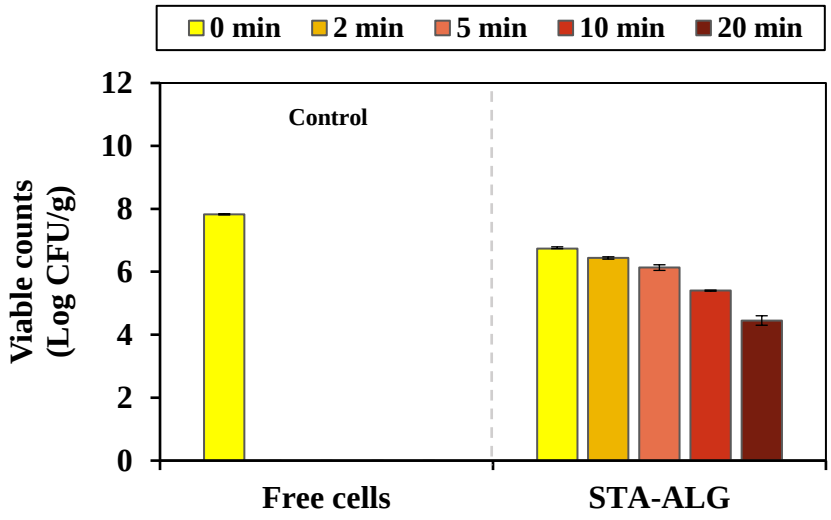


Figure 27. Viable bacterial count of unencapsulated and extrusion-formed, resistant starch-alginate (STA-ALG)-based encapsulated *B. lactis* Bb-12 after heat treatment at 60°C for 2, 5, 10 and 20 min

In accordance with my present results, Simpson et al. (2005) also reported high tolerance of spray-dried encapsulated *B. lactis* Bb-12 after treating them at several temperatures, including 60°C. Furthermore, resistant starch-alginate capsules provided even better protection for *B. pseudocatenulatum* G4 with a viability loss of only 0.85 logs after 30 min of heat exposure at 60°C, compared to my related results obtained for *B. lactis* Bb-12. In addition, in this same study, better heat tolerance was also observed for the *Bifidobacterium* strain than the *Lactobacillus* one (Teoh et al., 2011).

4.3 EVALUATION OF THE PROBIOTIC-LOADED CAPSULES PREPARED BY EMULSIFICATION TECHNIQUE

4.3.1 Preparation of emulsification-formed capsules

Following the extrusion technique, encapsulation of model probiotic *L. casei* 01 was also successfully conducted by emulsification method. This encapsulation was based on the external ionotropic gelation of probiotic-contained aqueous polymer phase emulsified within a larger volume of sunflower oil (continuous, dispersing) phase (by 50 mL) ; for the external gelation process, a proper cross-linking (aqueous) solution was added into this emulsion system. Considering the results I obtained previously with the extrusion-formed encapsulated probiotics, the emulsification-related encapsulation studies only focused on the preparation and evaluation of the resistant starch-reinforced alginate gel capsules. Besides, pure alginate gel capsules were also prepared by emulsification, and investigated as a control capsule type (without blending). Table 9 presents the applied exact composition of hydrogel polymer and cross-linking solutions needed for the successful emulsification-based formation of these gel capsules. Several investigations were subsequently carried out to examine their morphology, size distribution, encapsulation efficiency, and bacterial protection ability against heat stress and *in vitro* gastrointestinal conditions. To clearly distinguish from the other common type with internal gelation, this method will be occasionally referred later to as 'emulsification/external gelation' (or shortly to as 'em. / ext. gelation') in the following sections.

Table 9. Different variations of successfully prepared gel capsules, and the composition of hydrogel and cross-linking solutions used for their emulsification/external gelation-based preparation

Types of gel particles	Hydrogel solution (to be dispersed in emulsion system and to be gelled)		Cross-linking solution (added to emulsion system for gelation process)
	Component 1	Component 2	
Pure alginate	-	2% (w/v) Alginate	0.05 M $CaCl_2$
Starch-alginate blend	2% (w/v) Resistant starch	2% (w/v) Alginate	0.05 M $CaCl_2$

4.3.2 Morphology of the emulsification-formed capsules

The appearance of these em. / ext. gelation-formed gel capsules can be seen in Figure 28A and 28B. According to it, they could be characterised as highly variable in shape, some of which resembled either drop, oval, sphere, or fibre. Regardless of the type of encapsulant used, a considerably wider size distribution than that obtained for the extrusion-based particles was obtained, ranging roughly from 0.8 to 13 mm according to the measurement with ImageJ software. This was due to that the

droplet (i.e. final gel capsule) formation with this specific procedure relied on the dispersion in the emulsion system, rather than the fixed-sized capillary (needle) passage of polymer as was the case with the extrusion method. Thus, particles with more uniform size and shape may have been achieved with more thorough dispersion of the aqueous alginate-based polymer phase within the oil phase. In addition, the application of freeze-drying step on the prepared gel capsules was previously shown to be a good way for reducing particle size (Zou et al., 2011). As it was with the particles formed with the extrusion method, resistant starch-alginate particles distinguished from alginate ones by their opaque white colour, suggesting that the starch was successfully blended in the alginate matrix. Clumping of these particles was largely not observed, except when some excess oil still remained on the particles due to the insufficient washing process or the poor phase separation of the formed emulsion system. It should be also noted that the phase separation of the emulsion was a bit clearer when resistant starch was also included in the alginate polymer phase.

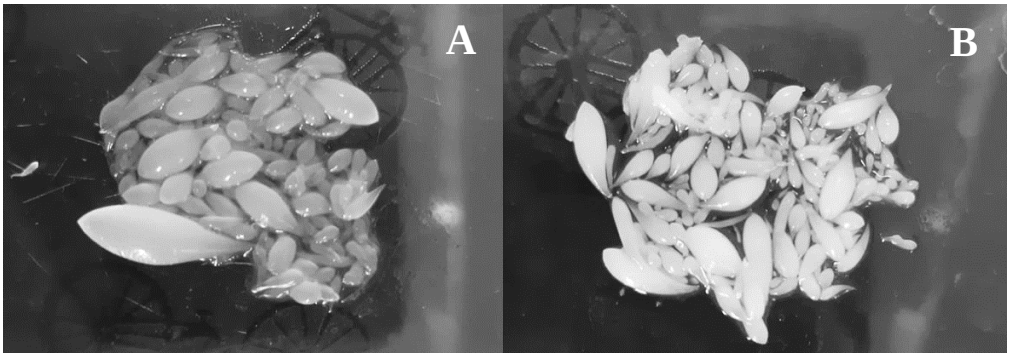


Figure 28. Appearance and morphology of the alginate (A) and resistant starch-reinforced alginate (B) gel capsules obtained by the emulsification/external gelation technique

4.3.3 Encapsulation yield of emulsification-prepared capsules containing *L. casei* 01

When *L. casei* 01 was encapsulated with alginate and resistant starch-alginate gel matrices using the em. / ext. gelation method, the encapsulation efficiency did not differ considerably between the two gel matrix variations, showing equally high efficiencies of 88.6 % and 89.9 %, respectively. Interestingly, extrusion-based encapsulation was found to give a lower yield than that by this emulsification technique; in that case, only 64 % and 77 % of total bacterial number were entrapped into alginate and resistant starch-alginate gel matrices, respectively (Section 4.2.4). The exact reason for this is uncertain but could be related to the possible leakage arising from the stirring (~ 200 rpm) used in the extrusion technique, during the resting state of formed gel droplets in the cross-linking

bath (for complete solidification of their gel matrix). In em. / ext. gelation method, this stirring step was not attempted during the gel droplet formation as it could easily disrupt the proper separation process between the oil and the aqueous polymer phases.

Comparing with the present results, Chen et al. (2012) reported a bit lower encapsulation yield of around 78% for alginate capsules formed with a similar external gelation type emulsification procedure as used in my study. In the case of externally gelled resistant starch-alginate capsules, higher encapsulation yield of 98.12 % was obtained for *L. casei* (Khosravi Zanjani et al., 2014). When internal gelation was applied during the emulsification-based encapsulation (for the gelation of dispersed polymer phase), Martin et al. (2013) reported a slightly higher yield of around 97% and a lower yield of around 74% for *L. fermentum* CECT5716 encapsulated in corn starch blended and pure alginate capsules, respectively. Zou et al. (2011), who performed encapsulation with the emulsification/internal gelation method along with subsequent freeze-drying, observed even far lower yields of 43–50% for alginate-based gel capsules even if they were blended either with starch or pectin, or coated with chitosan or poly-L-lysine. It can possibly be ascribed to the tiny particles sizes that resulted by the abovementioned freeze-drying step.

4.3.4 Viability of emulsification-formed encapsulated and free *L. casei* 01 exposed to simulated gastric and intestinal conditions

After the physical characterisations, the protection effect of the em. / ext. gelation-formed resistant starch-alginate gel capsules on *L. casei* 01 was also assessed with *in vitro* gastrointestinal fluids based on the protocol described by Krasaekoopt et al. (2004). Besides, pure alginate encapsulated and free *L. casei* 01 were also examined as an encapsulated and an unencapsulated control samples, respectively. As was in Section 4.2.11, the viability of these bacteria was assayed by treatment in solely 135 min of simulated gastric fluid (SGF) (including pepsin), solely 150 min of simulated intestinal fluid (SIF), and sequential 135 min of simulated gastric (SGF) and 150 min of simulated intestinal fluids (SIF). Figure 29 presents the survival profile of *L. casei* 01 as a function of treatment time, both for free and each (alginate, resistant starch-alginate) encapsulated cells.

The initial viable counts right before the treatment were 9.53 log CFU/g for free, 9.08 log CFU/g for resistant starch-alginate encapsulated bacteria, and 7.91 log CFU/g for alginate-encapsulated bacteria. Due to the low acid resistance, undetectable viable cells were found for free *L. casei* 01. However, this poor viability of *L. casei* 01 bacteria was observed even with encapsulation (in alginate and resistant starch-alginate capsules), after treated with both solely 135 min of SGF and sequential 135

min of SGF + 150 min of SIF conditions – retaining no viable counts in the end. In fact, viable counts were only detected when bacteria were exposed solely to the SIF for 150 min. In this case, resistant starch-reinforced alginate capsules provided the best protection against the bile salt medium, retaining the cell viability with an only loss of 2 logs, whereas alginate without resistant starch again failed to protect the bacteria.

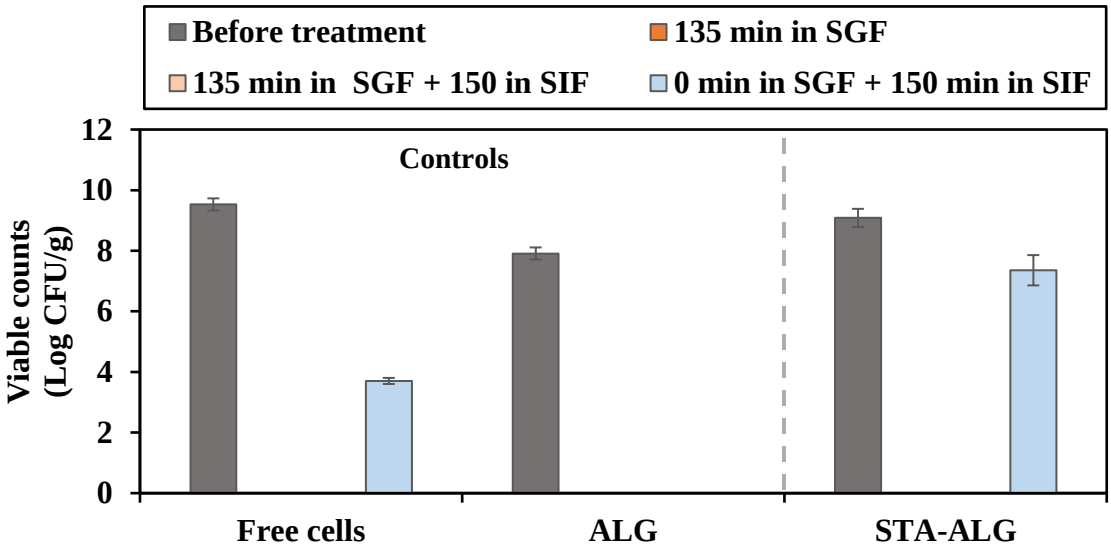


Figure 29. Survival of free and emulsification-based encapsulated *L. casei* 01 in alginate (ALG) and resistant starch-reinforced alginate (STA-ALG) capsules during exposure to simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)

The result regarding my emulsification-formed alginate capsules was similarly obtained by Ji et al. (2019), who also used emulsification method for encapsulating *B. longum* into pure alginate capsules, and exposed them separately to simulated gastric fluid (pH = 2.5) for 120 min and to simulated intestinal fluid (pH = 6.8) for 120 min. Furthermore, Khosravi Zanjani et al. (2014) demonstrated that the survival of the encapsulated *L. casei* ATCC 39392 could be improved by the addition of inulin into the starch-alginate gel matrix. They accessed survivability with separate treatments in simulated gastric fluid (pH = 1.5, including pepsin) and simulated intestinal fluid (pH = 8, including pancreatin and NaCl). In comparison, while the extrusion-formed alginate and especially resistant starch-alginate capsules (Section 4.2.6 and 4.2.7) provided good protection for *L. casei* 01 under the strong acidic conditions of gastric fluid, the emulsification-based encapsulation – even with resistant starch reinforcement of alginate matrix – fared way poorer in this aspect. With extrusion-formed resistant starch-alginate capsules the viability loss was observed as 3.79 log CFU/g after 135 min exposure to SGF (pH = 2, along with pepsin content). Although, the sequential treatments in SGF (135 min) (pH

= 2) and SIF (150 min) (pH = 7.43) was equally lethal to the bacteria even with extrusion-based encapsulation, neither in alginate nor resistant starch-alginate matrix.

4.3.5 Thermal stability of emulsification-based encapsulated and free *L. casei* 01 under different high-temperature conditions

Heat resistance study was also conducted with the emulsification-based encapsulated *L. casei* 01 in similar way as was in the extrusion-based encapsulation study (Section 4.2.8 and Section 4.2.12) but using only the 60°C water bath. Figure 30 presents the viability of unencapsulated, and encapsulated bacteria (in alginate and resistant starch-alginate) exposed to the heat-treatment at 60°C, as a function of treatment time (0, 2, 5, 10, 20 min). The initial viable cell counts prior to the heat exposure were 10.11 log CFU/g for the free cells, 7.85 log CFU/g for the alginate encapsulated cells, and 9.44 log CFU/g for the resistant starch-alginate encapsulated cells.

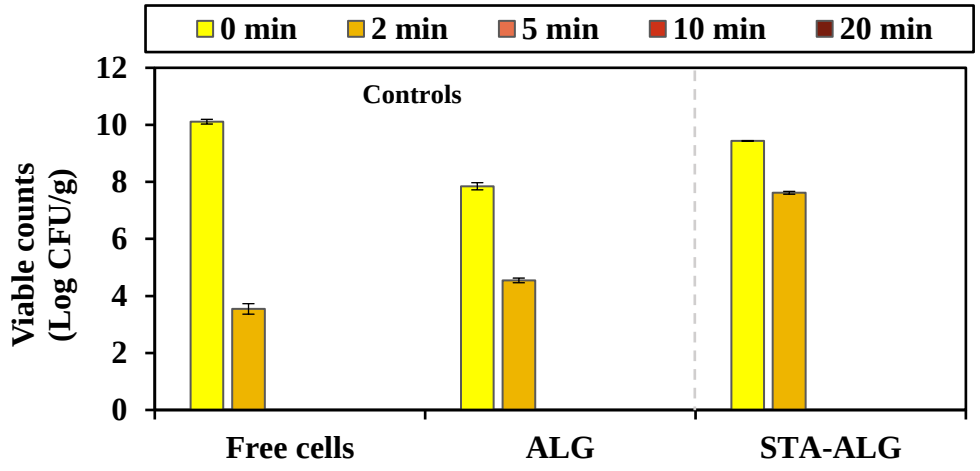


Figure 30. Viable bacterial count of free and emulsification-based encapsulated *L. casei* 01 in alginate (ALG) and in resistant starch-reinforced alginate (STA-ALG) capsules after heat treatment at 60°C for 2, 5, 10 and 20 min

After treated for 2 min in 60°C water bath, free *L. casei* 01 showed a viability loss of around 6.6 log CFU/g. However, this heat tolerance was improved by encapsulation, especially when resistant starch-alginate was used as encapsulants. In this case, the viability reduced by around 1.8 log CFU/g to 7.62 log CFU/g with the resistant starch-reinforced alginate capsules, and by around 3.3 log CFU/g to 4.54 log CFU/g with the pure alginate capsules. However, with the 5-min-long exposure, the viability of *L. casei* 01 reduced to an undetectable level in all cases whether they were encapsulated or not. In view to this result, *L. casei* 01 showed a bit better resistance to 60°C treatment when encapsulated by

extrusion method (Section 4.2.8). For instance, when encapsulation in resistant starch-alginate was applied, bacteria still survived the 5-min treatment (with a viability loss of 3.4 log CFU/g).

Xing et al. (2014), who also evaluated the heat tolerance of similarly encapsulated *L. acidophilus* (into porous starch-contained alginate capsules) in 60°C water bath, observed a better survival rate than that of *L. casei* 01 in this study, both with 10- and 20-min treatment. Also, better heat tolerance was shown by Ding and Shah (2007), who obtained an average decrease of 2 logs after 30-min heat treatment of different *Lactobacillus* strains (*L. rhamnosus, L. salivarius, L. acidophilus* and *L. paracasei*) at 65°C. They also showed enhanced heat tolerance with emulsification-based encapsulation (in alginate matrix) than without it.

4.3.6 Storage stability of emulsification-based encapsulated and free *L. casei* 01 in different non-dairy food matrices at different temperatures

The evaluation of long-term storage stability both in pre-fermented oat and beetroot drink was also conducted with *L. casei* 01 encapsulated by the em. / ext. gelation method. Following the earlier satisfactory performance in bacteria protection, only resistant starch-alginate encapsulated cells were involved in this storage experiment, along with free cells as controls. This storage was conducted over a period of 5 months, both at 4°C (refrigerated) and 20°C (ambient) temperatures, while viable cell counts in each oat drink were enumerated on a monthly basis. The initial viable counts prior to the storage period were detected as 9.61 and 9.05 log CFU/g for free and encapsulated cells, respectively.

In the case of both storages in oat and beetroot drink, the initial viable counts of free and resistant starch-alginate encapsulated *L. casei* 01 before the storage period were detected as around 8.30 and 9.02 log CFU/g, respectively.

4.3.6.1 Viability of encapsulated and free L. casei 01 in oat drink

The stability of free and encapsulated *L. casei* 01 in oat drink are shown in Figure 31A for 4°C and Figure 31B for 20°C storage temperature. The pH of fermented oat drink was measured as 3.5. By storing at cold (4°C) temperature, the viability of both unencapsulated and resistant starch-alginate encapsulated bacteria steadily remained above the recommended minimum level of log 6 CFU/g (Yao et al., 2020) throughout the whole 5-month period, averagely at 8.27 and 8.57 log CFU/g. In contrast, the storage stability appeared weaker at 20°C as neither of the oat drink varieties had viable counts left at the 4th month. Albeit, while the resistant starch-alginate capsules did still enhance the viability

of their encapsulated cells with a loss of about 1.60 logs, the free cells lost nearly two third of their initial viable counts by the 3rd month of storage. These viability results also revealed that as long as the storage in oat drink had been examined for short period of maximum 2 months, no significant stability difference would have been observed between the free and the encapsulated bacteria.

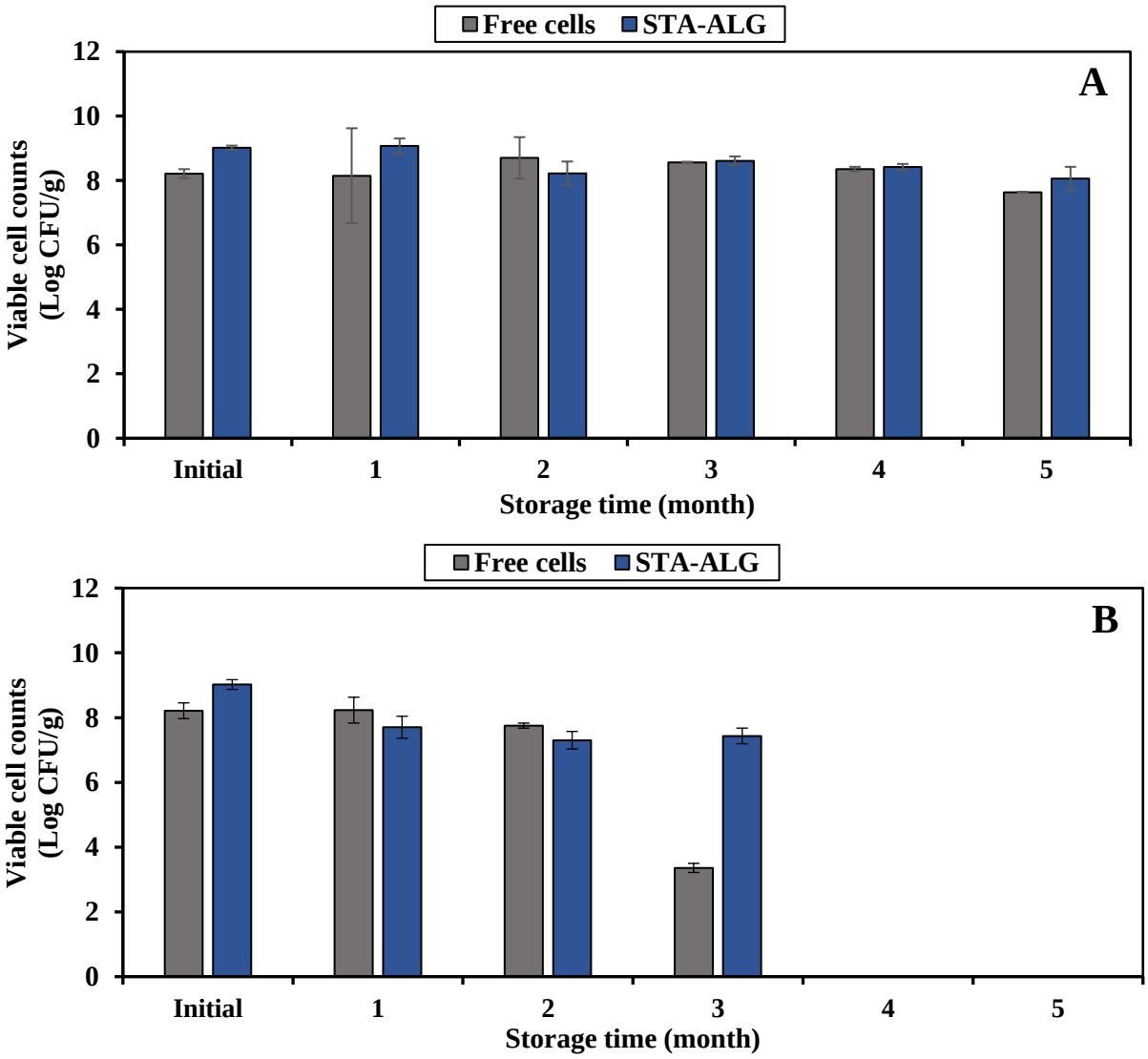


Figure 31. Viability of free and emulsification-based encapsulated *L. casei* 01 in resistant starch – alginate (STA-ALG) capsules over the 5-month storage in oat drink at 4°C (A) and 20°C (B). 'Initial' refers to the viable cell counts detected right before the storage experiment but after the fermentation step

In comparison with the extrusion-encapsulated bacteria (Section 4.2.9.1), it was seen that this type of encapsulation did not considerably alter the storage stability of *L. casei* 01 in overall. The only notable difference was that some viable cells could be still detected after 4 months of storage at 20°C with extrusion-based encapsulation, unlike with the emulsification-based one.

Largely same degree of bacterial protection (*L. plantarum*) was reported in an earlier study for similarly formed resistant starch-alginate capsules over 8 weeks of refrigerated (4°C) storage in yogurt, which resulted in enhanced stability relative to the unencapsulated cells (Shafiei, 2018). Sultana et al. (2000), who also similarly encapsulated their *L. acidophilus* 2401 and *B. infantis* 1912 in Hi-Maize starch-blended alginate capsules and then stored them in yogurt with a less acidic pH (4.6), observed a similar viable cell reduction (0.5 log CFU/g) as that found in the present study for *L. casei* 01 over the period of 8 weeks (~ 2 months). They also demonstrated a 1 log reduction in the viability of co-encapsulated and free cells over the same storage period.

4.3.6.2 Viability of encapsulated and free L. casei **01** *in beetroot drink*

Figure 32A and Figure 32B presents the viability of free and encapsulated *L. casei* 01 in beetroot juice stored at 4°C and 20°C. Before this storage period, the pH of fermented beetroot juice was 4.1. This storage study showed that the protective effect of the emulsification-based encapsulation was clearly apparent on the storage stability of *L. casei* 01 in beetroot juice not only at 20°C (as expected), but even at 4°C. In the case of cold storage (4°C), the viable counts of encapsulated bacteria rather stabilised, whereas the one of free bacteria showed decreasing trend over the whole storage period. Furthermore, under ambient conditions (20°C), the shelf-life of bacteria was extended by additional 2 months with the encapsulation, that is, their viable counts were still detected even after 5 months of storage unlike the free bacteria. Although, they ended up at much lower viable level of around 3.94 log CFU/g as compared to their initial viable counts before storage period.

Similar pattern was also observed for the extrusion-based encapsulated *L. casei* 01 (Section 4.2.9.3), since in that case, the poor adaptation of free cells to the beetroot juice was also improved by using encapsulation with resistant starch-alginate gel matrix at both ambient and refrigerated storage temperatures. Also, this improvement was only seen when the storage was performed for at least 3 months.

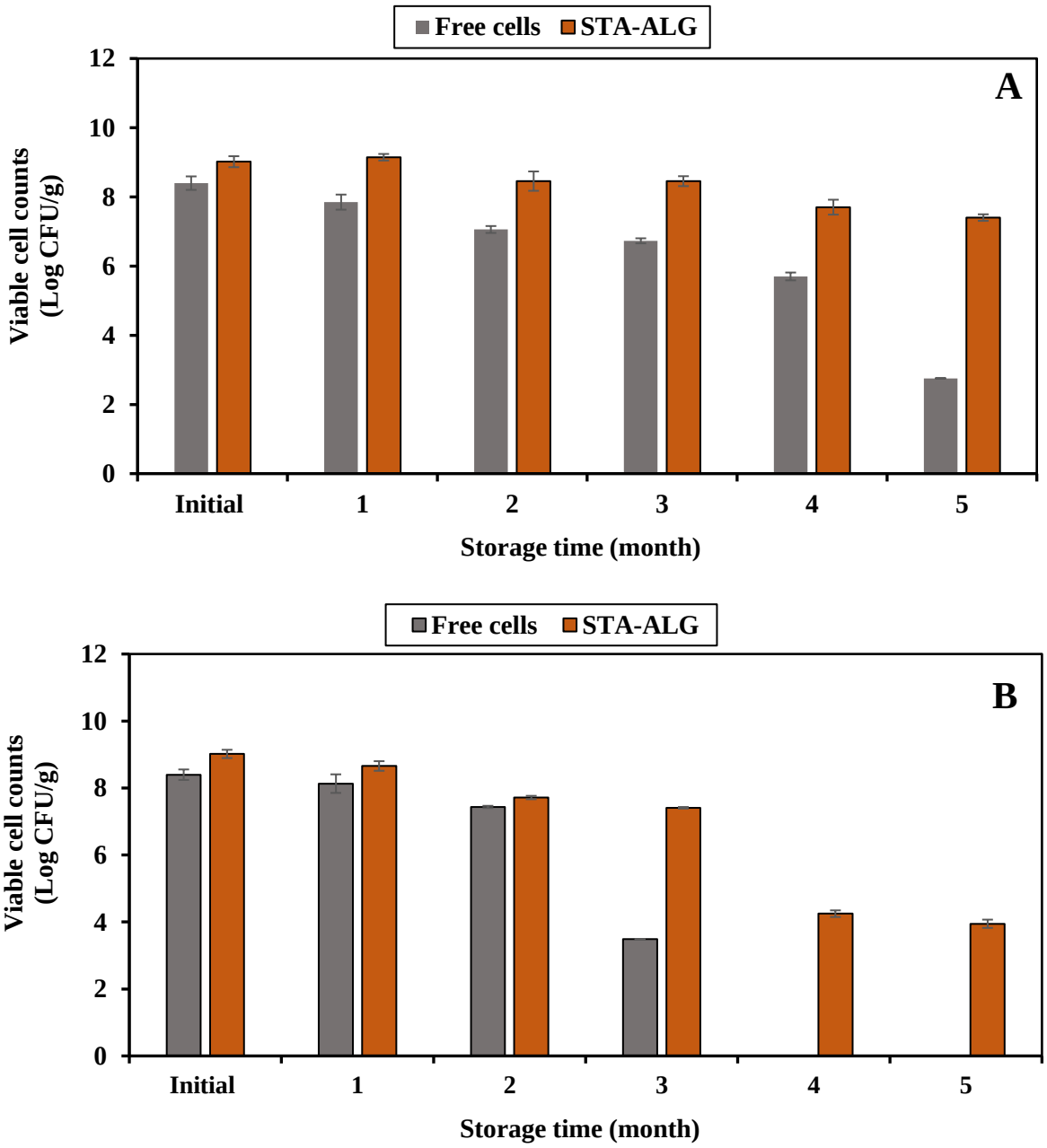


Figure 32. Viability of free and emulsification-based encapsulated *L. casei* 01 in resistant starch – alginate (STA-ALG) capsules over the 5-month storage in beetroot drink at 4°C (A) and 20°C (B). 'Initial' refers to the viable cell counts detected right before the storage experiment but after the fermentation step

4.3.7 Preparation and encapsulation yield of emulsification-formed capsules containing *B. lactis* Bb-12

The effect of the em. / ext. gelation-based encapsulation was also evaluated on the viability of a bifidobacterial strain. To this end, *B. lactis* Bb-12 were encapsulated with the same procedure as that

performed for the *Lactobacillus* strain, and then investigated similarly for their resistance to heat stress and *in vitro* gastrointestinal conditions. However, resistant starch-alginate capsules were applied and investigated alone in this study, which were prepared using the same compositions as in Table 9 (in Section 4.3.1). As a result of this encapsulation, viable *B. lactis* Bb-12 was successfully entrapped with a yield of 92 %, which turned to be a bit higher than what obtained for the case of *L. casei* 01 encapsulation in resistant starch-alginate (90 %) (Section 4.3.3). This encapsulation yield was also a bit higher than that reported by Holkem et al. (2016), who also encapsulated *B. lactis* Bb-12 with emulsification method, but into internally gelled alginate capsules. In this case, emulsification/internal gelation also resulted an encapsulation yield of 90 %. However, the present efficiency was shown to be much lower than that found in the study of Khosravi Zanjani et al. (2014), who also encapsulated *B. bifidum* into externally gelled resistant starch-alginate matrix and obtained an encapsulation efficiency of 98 %. With respect to encapsulation efficiency, there was no considerable difference found between this type of encapsulation and the extrusion-based one (Section 4.2.10).

4.3.8 Viability of emulsification-formed encapsulated and free *B. lactis* Bb-12 exposed to simulated gastric and intestinal conditions

The survival of free and resistant starch-alginate encapsulated *B. lactis* Bb-12 under simulated gastrointestinal conditions is illustrated in Figure 33. This survival study – as the one with *L. casei* 01 – was conducted with in three ways: separate treatments in simulated gastric fluid (SGF) (including pepsin) and in simulated intestinal fluid (SIF), and also sequential treatment in these two simulated fluids. Free cells as control showed extreme susceptibility to these strong acidic (pH = 2), pepsin activity and bile salt conditions, with their viable cells decreasing to undetectable levels from the initial counts of 7.50 log CFU/g (right before the survival assay). On the other hand, the bacteria encapsulated in resistant starch-alginate capsules were found to highly maintain their viable counts with a minimal loss under the bile salt conditions of SIF. Interestingly, the bacteria lost all of their viability even with the encapsulation when exposed to SGF alone and sequential SGF and SIF, so did those in free form.

When emulsification involved internal gelation, a small ratio of initial *B. bifidum* F-35 bacteria still remained viable even at the end of the 2-h treatment in SGF (Zou et al., 2011), and they also encapsulated bacteria into starch-reinforced alginate capsules. This may be ascribed to the internal gelation process, with which the cross-linker Ca^{2+} -ions diffuse outward within the polymer matrix; this may form more solid gels than with external (inward) gelation. However, this higher survival rate

in that study is somewhat surprising as it has been demonstrated that the acetic acid – exclusively applied for the internal gelation – can weaken the physical and functional characteristics of the starch (Majzoobi & Beparva, 2014).

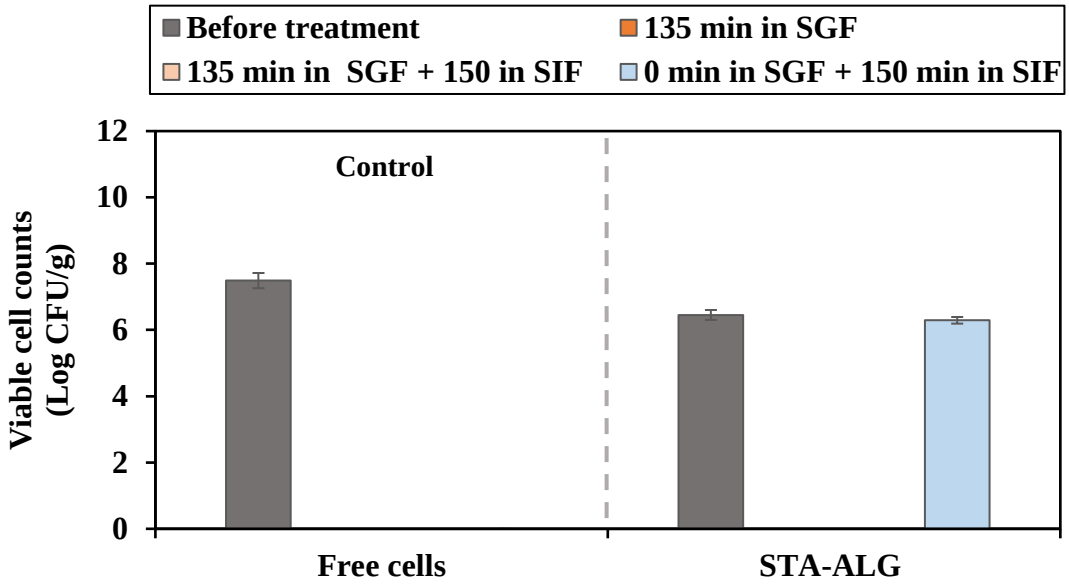


Figure 33. Survival of free and emulsification-based encapsulated *B. lactis* Bb-12 in resistant starch-reinforced alginate (STA-ALG) capsules during exposure to simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)

Overall, considering the present results with the *Bifidobacterium* strain and also with the *Lactobacillus* one above (Section 4.3.4), em. / ext. gelation-formed capsules (specifically with resistant starch-alginate gel matrix) was found to fare poorly in the viability protection against the low pH and pepsin conditions of SGF and against the combined effect of SGF and SIF with bile salts. However, they generally provided much improved tolerance to bacteria when exposed solely to the bile salt conditions as compared to the free cells. When these bacteria were encapsulated with the extrusion technique (Section 4.2.6 and 4.2.11), higher tolerance towards either SGF or combined conditions of SGF and SIF was generally observed than those encapsulated with the em. / ext. gelation. This more or less corroborates with the results reported by Muthukumarasamy et al. (2006), who also compared these two types of method for encapsulating *L. reuteri*. The poorer protection obtained with the

emulsification-based encapsulation may be attributed to some degree of loosening effect of oil on the alginate gel strength when applied during the encapsulation process (Chan, 2011).

4.3.9 Thermal stability of emulsification-based encapsulated and free *B. lactis* Bb-12 under different high-temperature conditions

The effect of heat exposure on the em. / ext. gelation-based resistant starch-alginate encapsulated *B. lactis* Bb-12 bacteria were finally assessed using a water bath at 60°C, for which the viability was determined after several treatment times (Figure 34). The obtained data revealed that the encapsulated bacteria – although suffered some degree of viability loss – could still be detected after 5 min of heat treatment. In this case, the remaining viable counts was observed as 3.37 log CFU/g, decreasing from the initial counts of around 6.89 log CFU/g. In contrast, free cells as control lost all of their viability even within 2 min of treatment, from their initial counts of around 7.83 log CFU/g, thereby indicating improved heat tolerance with the encapsulation than without it. In a previous study, the viability of *B. longum* loaded in alginate and in chitosan coated alginate capsules – with the same encapsulation method – showed much less sensitivity to 60°C heat-treatment even for 30 min, with a minimal average loss of 0.58 log CFU/g, respectively (Ji et al., 2019). Furthermore, Ding and Shah (2007) found an only average decrease of 1.67 log for bifidobacterial strains (*B. longum*, *B. lactis* Bi O4, *B. lactis* Bi 07) after 30 min of heat treatment at 65°C, using solely alginate encapsulation. Also, they reported improved heat tolerance with encapsulation than without it, although, there was no significant difference found after 60 min of treatment. All in all, this indicates the highly strain-specific attribute of heat tolerance.

Overall, this result also demonstrated that *L. casei* 01 bacteria in the same resistant starch-alginate capsules were generally more sensitive to this 60°C temperature conditions than the present bifidobacterial strain. This better heat tolerance with bifidobacteria was also largely found in the study of Ding and Shah (2007), in which emulsification method was also used for encapsulation. Furthermore, it was also the case with extrusion-based encapsulation, with which *B. lactis* Bb-12 exhibited, in fact, even more tolerance in a way that their viable cells (~ 4.5 log CFU/g) were still retained even after 20 min treatment (Section 4.2.12).

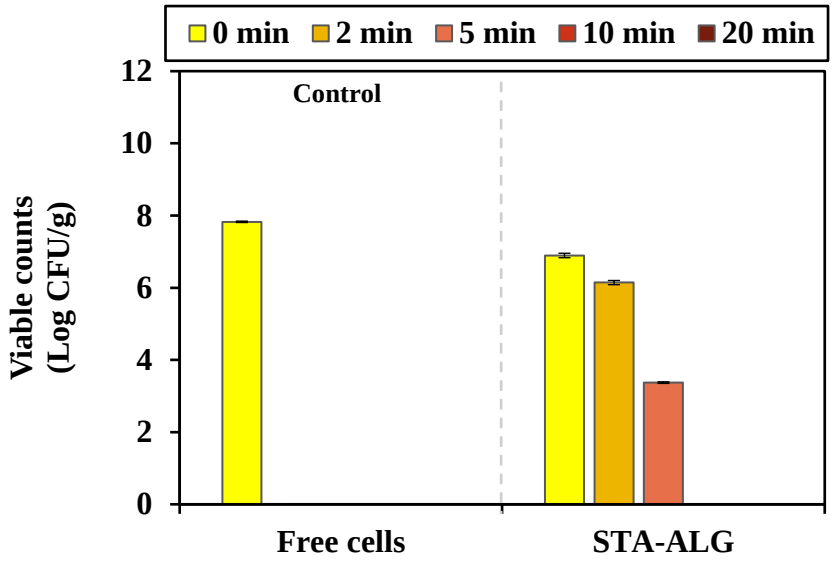


Figure 34. Viable bacterial count of free and emulsification-based encapsulated *B. lactis* Bb-12 in resistant starch-reinforced alginate (STA-ALG) capsules after heat treatment at 60°C for 2, 5, 10 and 20 min

4.4 EVALUATION OF THE PROBIOTIC-LOADED CAPSULES PREPARED BY ELECTROSPRAYING TECHNIQUE

4.4.1 Preparation of the electrospray-based capsules

Probiotic encapsulation was also successfully performed by electrospraying technique into different polymeric variations of gel microparticles. In specific, pure alginate and resistant starch-alginate blend gel particles were produced by electrospraying itself, and the pure alginate particles were additionally coated with chitosan as a third type of particles. The exact compositions of hydrogel, cross-linking and chitosan-based coating solutions employed for the preparation of these particles are detailed in Table 11. The resultant probiotic formulations (i.e. capsules) were subsequently examined in several physical aspects like morphology, size distribution and mucoadhesive properties, and also in some physiological aspects like encapsulation yield and viability of encapsulated probiotics under *in vitro* gastric conditions. It should be noted that *L. plantarum* NCDO 1752 was applied and encapsulated as a model probiotic strain in this electrospraying study.

Table 11. Composition of hydrogel and cross-linking solutions used for electrosprayed-based preparation of different gel particles

Types of gel particles	Hydrogel solution (to be electrosprayed)		Cross-linking solution	Coating solution
	Component 1	Component 2		
Pure alginate	-	2% (w/v) Alginate	0.05 M $CaCl_2$	-
Starch – alginate blend	2% (w/v) Resistant starch	2% (w/v) Alginate	0.05 M $CaCl_2$	-
Chitosan coated alginate	-	2% (w/v) Alginate	-	0.2 % (v/w) Chitosan

4.4.2 Morphology and size distribution of the electrospray-based capsules

Fluorescence microscopy images of the particles prepared using the different polymeric constituents are presented in Figure 35. These microscopic observations confirmed that the electrospraying process yielded spherically shaped particles. A chitosan layer was successfully formed on the alginate bead surfaces, with an average thickness of 18.5 μm; this was measured using the ImageJ analysis software. The laser light diffraction analysis (Figure 35) revealed that alginate microparticles were produced with a wide size distribution ranging from 30 to 600 μm (and span = 1.069, indicating the width of the distribution) and with the greatest part (12.95 %) of the microparticles population measured at 310 μm. This wide size range can be caused by the low viscosity of sodium alginate solution applied for the microcapsule formation (Zaeim et al., 2017). Very similar size distribution (and range) of alginate microcapsules has also been generated with the emulsion-based formation technique (Dikit et al.,

2015). The wide distribution shifted to a greater size range of 60 – 1300 µm (and a width of span = 1.060) when chitosan coating was applied on the alginate microparticles. In this case, the most frequent size (12.39%, similar to the alginate microcapsules without coating) detected in the whole microparticle distribution increased to 586 µm (Figure 35). Based on the volume (or mass)-based mean diameter value 'D (4,3)', derived from the centre of the volume (or mass) distribution (Resch-Genger, 2008), the mean sizes of the whole particle population are estimated to be 309 µm and 607 µm for uncoated alginate and coated alginate particles, respectively. However, it is worth mentioning that some swelling and thus size expansion could occur while the alginate microparticles were stirred in the chitosan solution for the coating process; this could be attributed to the acidic conditions of the chitosan solution (pH = 2-3), similarly to what reported in a previous study (Cook et al., 2011). Furthermore, both particle size distribution curves as seen in the volume density plot (Figure 35) showed that the resultant particle size was not evenly distributed in the population as they spread out more towards the larger size range. A bimodal distribution is seen especially for the uncoated microparticles.

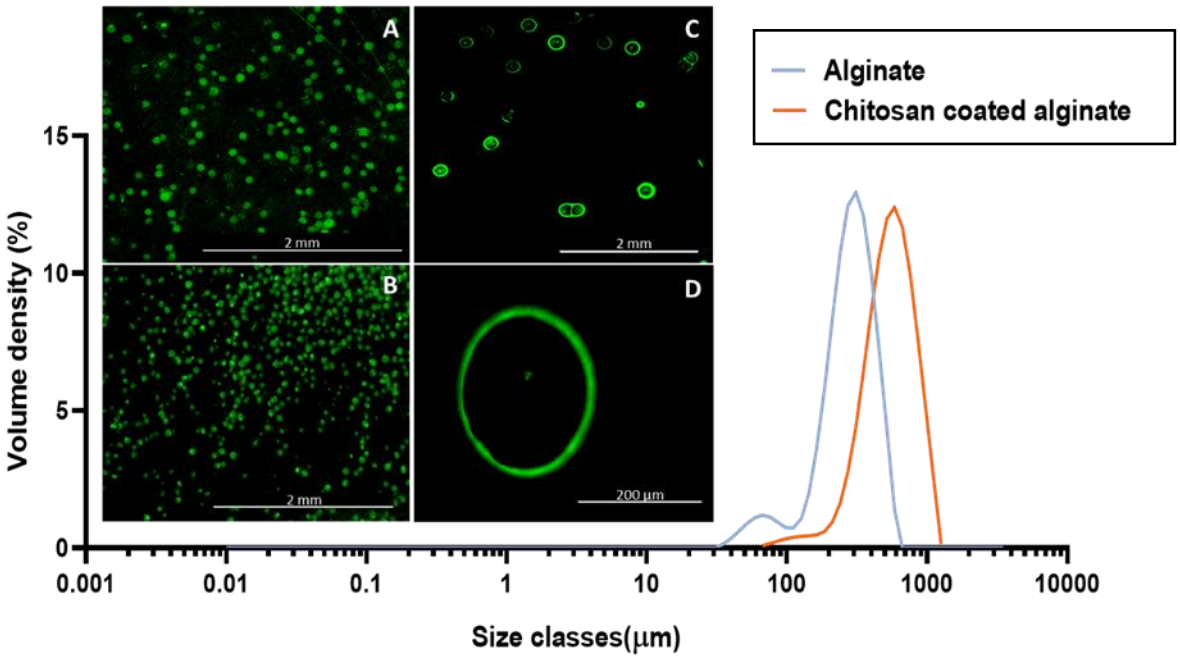


Figure 35. Particles size distribution of alginate and chitosan coated alginate microcapsules prepared with electrospray technique. Inserts show fluorescent microscopy images representing alginate (A), resistant starch-alginate (B) and chitosan coating layer on alginate (C and D) particles. Applied magnifications (and scale bars): 0.8x (2 mm) for image A, B, C and 8x (200 µm) for image D

Such small sized microcapsules prepared with the electrospraying method are needed when they are intended to be incorporated into food products, as too large sizes can negatively affect the sensory and textural characteristics (generating some undesirable grittiness feeling) of the certain food (Gbassi & Vandamme, 2012). If a post-drying (e.g., freeze- or spray-drying) step is applied the particle size can be further reduced, although, at the same time, this might result in a lower bacteria-loading yield, aggregation and cracking of the capsule gel matrix (Cook et al., 2012; Dianawati et al., 2016).

The morphology related results above are in agreement with the images generated using Morphologi 4 system (Figure 36) i.e., all particles have spherical shape and uniform size distribution. It should be noted that only uncoated alginate is shown in Figure 36 as its micrograph was better illustrative of the difference between uncoated and coated capsules.

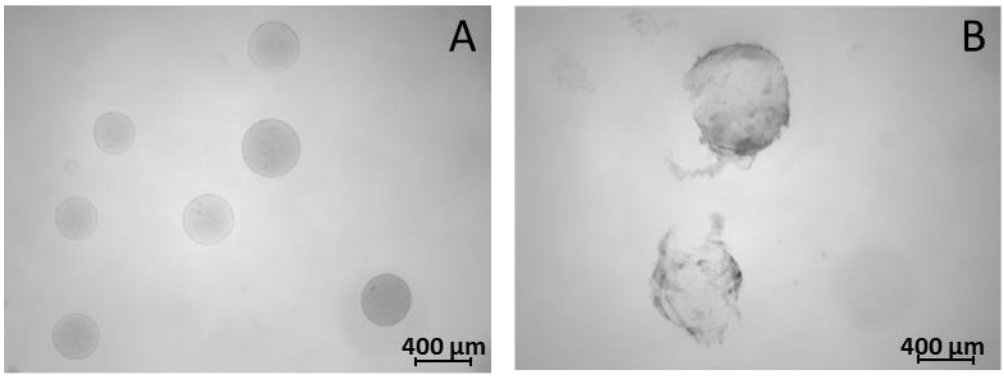


Figure 36. Light microscopic images (scale bar = 400 µm) derived from Morphologi 4 automated particle image analyser for uncoated (A) and chitosan coated alginate (B) particles (which prepared with electrospray technique)

4.4.3 Encapsulation yield of electrospray-based microcapsules containing *L. plantarum* NCDO 1752

The initial cell count of *L. plantarum* prior to electrospraying was 8.94 ± 0.12 log CFU/mL. Significant ($p < 0.05$), but slightly less (~1.16 log CFU/mL) viable bacterial count could be detected in the microcapsules produced right after the electrosprayed-based microencapsulation. As can be seen in Figure 37, the incorporation of resistant starch did not affect the encapsulation yield of alginate-based microcapsules significantly. In particular, the microcapsules with alginate showed a decrease by 1.25 log CFU/mL (86 ± 1.5 %), while resistant starch-reinforced alginate particles resulted in a viability loss of 1.06 log CFU/mL (88 ± 2.3 %)

Gómez-Mascaraque et al. (2017), who encapsulated bacteria by electrospraying, achieved a greater bacterial survival of 94% for *L. plantarum* with inclusion of acidified gelatine-whey protein

concentrate. However, they used coaxial approach and their encapsulation efficiency was lower compared to my results. In the case of other microencapsulation techniques, the encapsulation efficiency was generally found to be even higher on average with extrusion method (around 97 %) and with encapsulation in calcium alginate matrix [2 % (w/v)] (Afzaal et al., 2019; Gul & Dervisoglu, 2017; Lotfipour et al., 2012). Moreover, bacterial survival of between 74% and 98 % was yielded after spray drying-based microencapsulation with mixed alginate and soy protein isolate (Hadzieva et al., 2017). However, a comparable result as with the present resistant starch- alginate microcapsules could be found for those formed with the emulsification method using calcium alginate (~ 90 %) (Gul and Dervisoglu, 2017). Here, the low yield especially for the alginate-entrapped bacteria resulted with electrospraying technique may be due to their potential sensitivity to the combined stress effect of the high voltage electric field, rapid water evaporation and high shearing force operated throughout the whole encapsulation process (Coghetto et al., 2016).

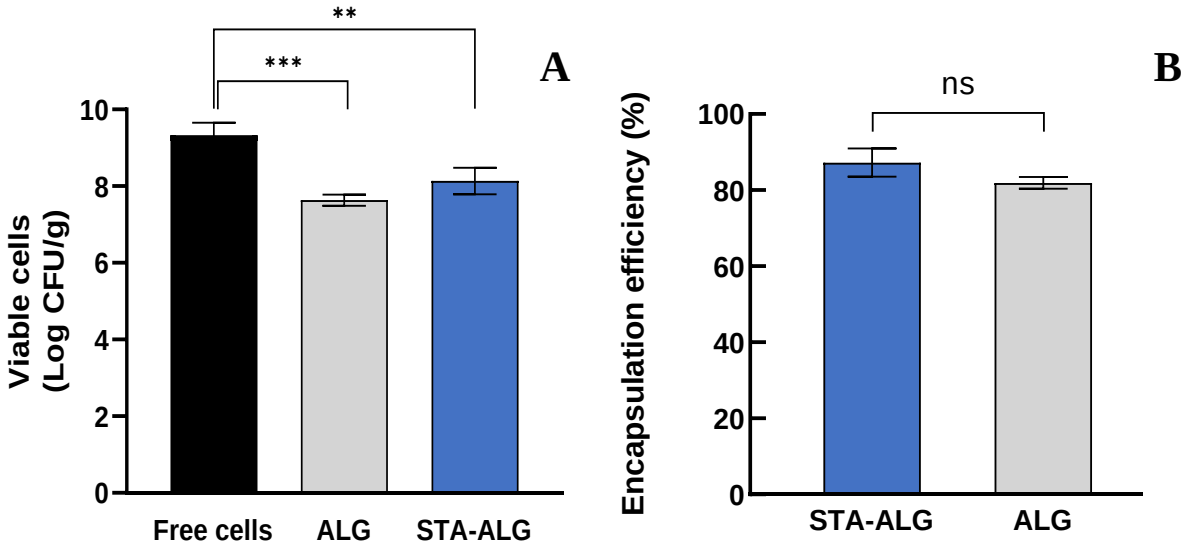


Figure 37. Number of bacterial cells survived after the microencapsulation process by electrospraying procedure (A) and the calculated percentage yields (B) of survived cells encapsulated in alginate (ALG) and resistant starch-alginate (STA-ALG) microcapsules. Significant differences are denoted by ** ($p < 0.01$) and 'ns' signifies no significant differences ($p > 0.05$)

4.4.4 Viability of electrospray-based microencapsulated and free *L. plantarum* NCDO 1752 exposing to simulated gastric condition

The viability results of free and microencapsulated *L. plantarum* in simulated gastric fluid (SGF) over different exposure times are summarised in Figure 38. In view of these results, the microcapsules prepared by electrospraying provided significantly enhanced survival rates ($p < 0.05$) for bacteria

within all formulations as no free cells were found even after 1 h of incubation, initiating from viable counts of log 8.14 CFU/mL. Pure alginate microcapsules significantly underperformed in terms of bacterial protection, compared to other types of alginate-based microcapsules with either blending with resistant starch or chitosan coating ($p < 0.01$). Electrosprayed resistant starch-alginate and chitosan-coated alginate formulations retained the viability of *L. plantarum* with lower losses, i.e. by 0.76-2.14 log CFU/mL and 0.49-3.68 log CFU/mL after 1 h and 2 h in SGF compared to alginate microparticles, respectively. Enhanced viability with chitosan coated alginate microcapsules could arise from the decreased pore size of the alginate gels after applying the coating layer (Pestovsky and Martínez-Antonio, 2019); this could, in turn, limit the contact of the bacteria with the gastric fluid. Interestingly, incorporating resistant starch into the alginate matrix resulted in a statistically similar protection with chitosan coating throughout the simulated digestion experiment. The former one could be explained by the direct presence of the resistant starch component within the alginate matrix, which may serve as a carbon source for the probiotic bacteria (Sultana et al., 2000; Zaman & Sarbini, 2015) or/and as an material that may decrease pore size of the alginate gel microcapsules. No notable alginate gel matrix disintegration – as with the extrusion and emulsification types of gel capsules – was observed for any formulations after the end of the gastric incubation, which can be related to that alginate exhibits an acid gel attribute at pK_a below ~ 3.5 (Nualkaekul et al., 2012; Onsoyen, 1992).

Zaeim et al. (2017), who likewise assessed electrospray-based microencapsulation of *L. plantarum* in alginate and consecutive coating with chitosan, showed similar viability reductions of around 2 and 3 log CFU/mL after 1 and 2 h gastric (pH = 2.5) exposure, respectively. In another paper from the same authors, chitosan coated alginate microcapsules, additionally incorporated with resistant starch, were reported to give a slightly weaker protection for *L. plantarum* than my present resistant starch-contained microcapsules during the 2 h simulated gastric treatment (Zaeim et al., 2019).

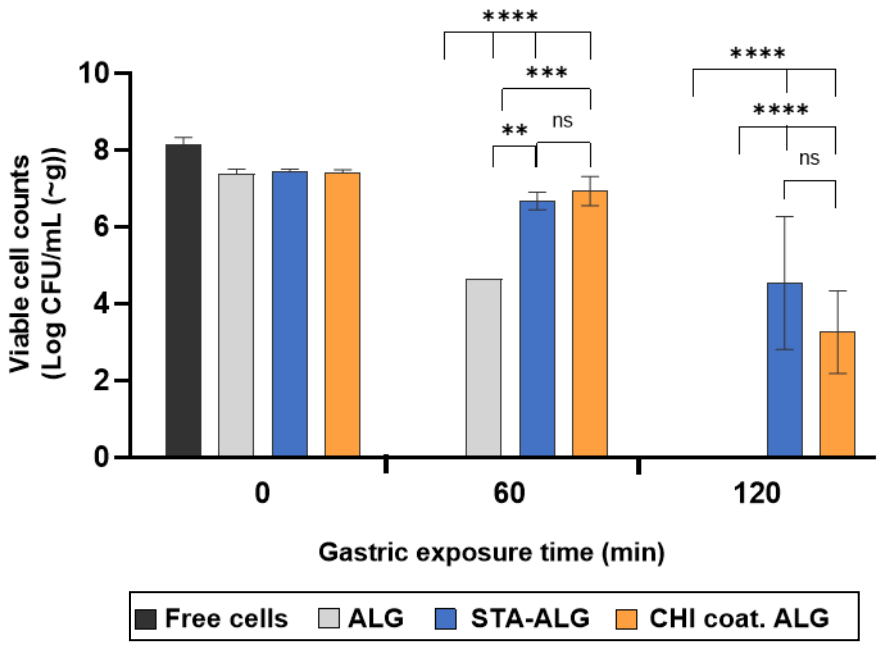


Figure 38. Viable numbers [CFU/mL (~g)] of free and electrospray-based microencapsulated *L. plantarum* bacteria in different variations of microcapsules over 2 h of exposure to simulated gastric fluid at 37°C. Significant p-values are denoted by ** ($p < 0.01$), * ($p < 0.001$), **** ($p < 0.0001$) and ns ($p > 0.05$, indicating non-significant differences). Abbreviations: ALG, alginate; STA-ALG, resistant starch-alginate; CHI coat. ALG, chitosan coated alginate capsules**

It has been reported in several studies (Argin, 2007; Chandramouli et al., 2004; Cook et al., 2012; Lee & Heo, 2000; Muthukumarasamy et al., 2006) that capsule size could be a significant factor for their efficiency in bacteria protection against high acidic conditions (e.g. in stomach); more specifically, enhanced viability can be achieved with increased capsule size. However, achieving a more micron-sized capsules is generally more preferred when it comes to utilising them as functional components in most food products. My results were found only partially in agreement with this hypothesis. Firstly, the alginate capsules with a bigger size obtained by the extrusion method provided roughly the same degree of bacteria protection in SGF (without the pepsin activity) (Section 4.2.6). In fact, in the case of resistant starch-alginate capsules, a smaller overall loss of viability could be observed with the much tinier capsules prepared by electrospraying than the bigger ones by extrusion. This indicates that the presence of resistant starch (or even some other prebiotics) in the gel matrix can nevertheless improve the protection ability of the tinier (micron-sized) capsules at least in gastric conditions. However, it should be noted that the examined probiotic strains in these two encapsulation studies were different (*L. plantarum* NCDO 1752 and *L. casei* 01), though it may be still relevant as both are

Lactobacillus species. Furthermore, both the electrospraying- and extrusion-formed capsules were prepared with the same compositions of alginate / resistant starch-alginate hydrogel solution. Comparing these results achieved for electrosprayed capsules with some other's results, Nualkaekul et al. (2012), using the extrusion method, observed slightly smaller losses in the bacterial population of *L. plantarum* of 2 and 3.8 log CFU/mL for alginate capsules after 1 h and 2 h of gastric exposure time (pH = 1.5) with having a much greater capsule size of around 3 mm. Besides, losses of 1.2 (1 h) and 2.5 (2 h) log CFU/mL were obtained with chitosan coated alginate hydrogels. A comparable protection of bigger alginate formulations was found in another study with extrusion techniques, however, emulsification-formed ones provided better protection there with even smaller sized capsules (Muthukumarasamy et al., 2006).

4.4.5 Mucoadhesive characteristics of electrospray-based microcapsules on gastric mucosa

Aside from keeping an adequate bacterial survival rate, several studies have also highlighted the importance of mucosal retention of microcapsules within the gastrointestinal tract for appreciably longer time, in the context of designing effective delivery systems for probiotics (Alli et al., 2011; Cook et al., 2012; van Tassell & Miller, 2011). For instance, retention on gastric epithelium may potentially improve the chance of some probiotics to curb gastric ulcers and gastric cancer diseases induced by *Helicobacter pylori* and to contribute gastric mucosal barrier protection. Furthermore, it is also reported that gastric mucus itself can provide an additional potential protective function for gastric survival of probiotics (Butel, 2014; Khoder et al., 2016; Koga et al., 2019; Singh et al., 2012). Accordingly, the mucoadhesive properties of unloaded alginate, resistant starch-alginate and chitosan-coated alginate microparticles were evaluated using an *in vitro* fluorescence imaging-based flow-through test on *ex vivo* porcine gastric epithelial mucosa, following the protocol applied previously by Cook et al. (2018), Kaldybekov et al. (2018) and Porfiryeva et al. (2019).

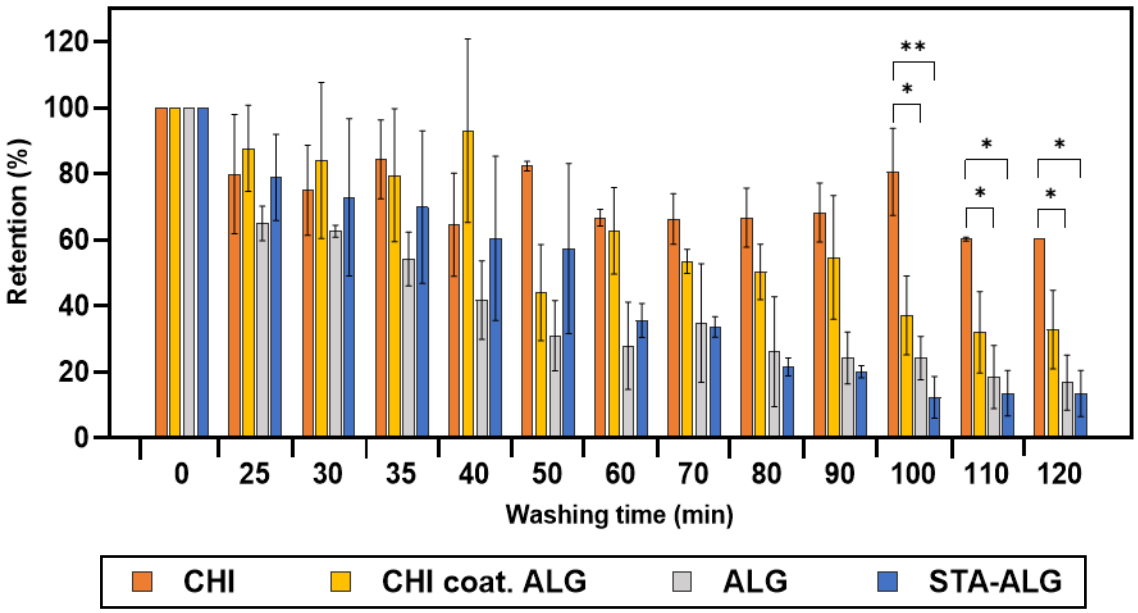


Figure 39. *In vitro* retention profiles of each microcapsule variation on ex vivo porcine gastric mucosa over 2 h of washing process with simulated gastric fluid. Statistical differences are denoted by * ($p < 0.05$) and ** ($p < 0.01$). The capsules were prepared with electrospray technique. Abbreviations: CHI, chitosan; CHI coat. ALG, chitosan coated alginate; ALG, alginate; STA-ALG, resistant starch-alginate capsules

The retention on the mucosa was observed based on the detected intensity of the fluorescent particles labelled with the agents mentioned in Section 3.3.7. To ensure the excitation of the fluorophores a portable UV LED flashlight torch was applied. SGF with pH = 2 was used to wash the particles off the mucosal surface. To avoid the leakage of fluorescent tracers, both alginate and resistant starch-alginate particles were labelled with the greater molecular weight FITC-dextran instead of Na-Fluo. A positive control experiment was also undertaken with pure chitosan particles (prepared as described in Section 3.3.3.4) as it is well known that chitosan exhibits strong mucoadhesive properties due to its cationic nature (Khutoryanskiy, 2011).

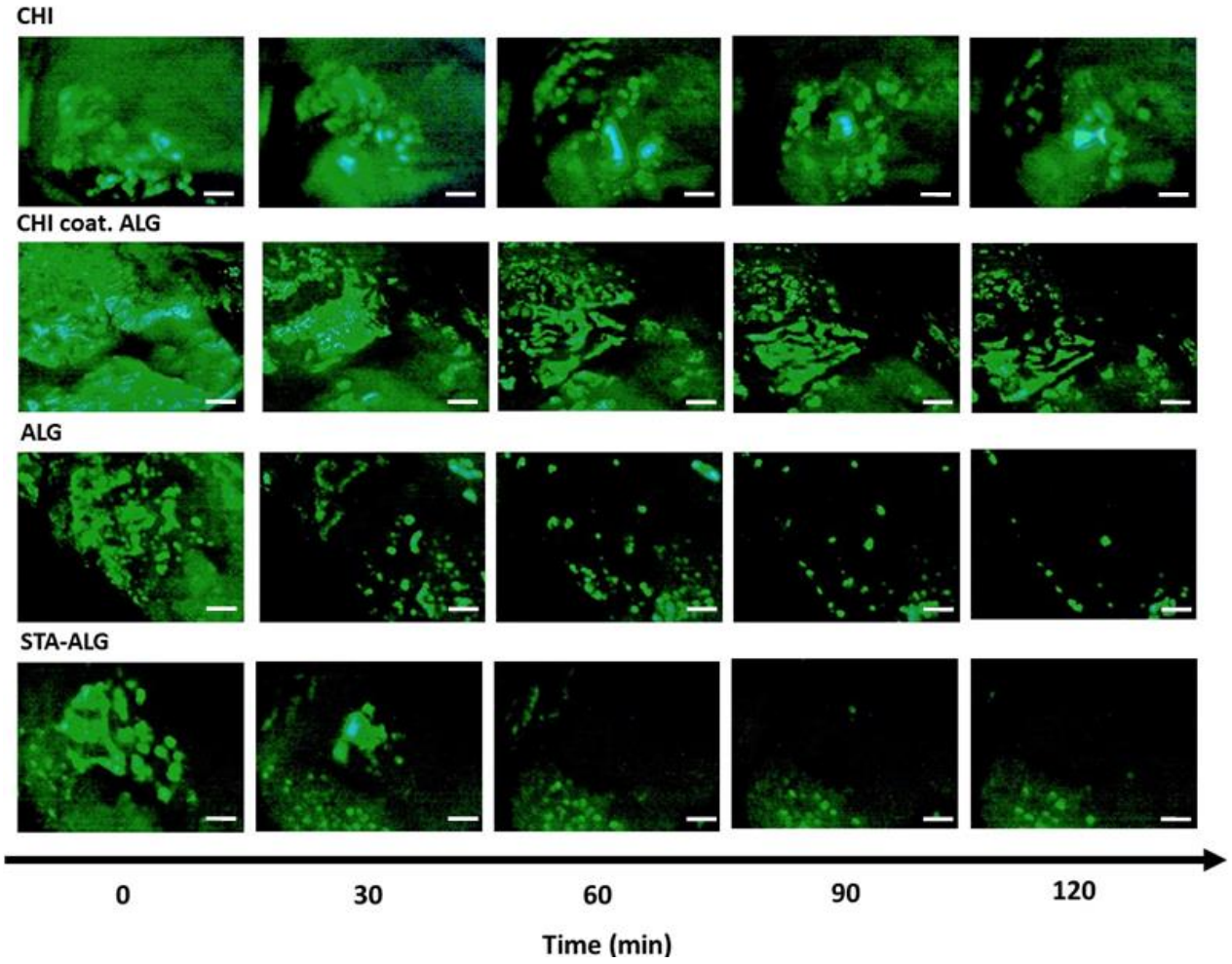


Figure 40. Example fluorescence images (40x) showing retention of each variation of microcapsules (prepared with electrospraying) on porcine gastric mucosa after the indicated time of washing with simulated gastric fluid (0.2 % (w/v), pH = 2). Abbreviations: CHI, chitosan; CHI coat. ALG, chitosan coated alginate; ALG, alginate; STA-ALG, resistant starch-alginate capsules. Scale bar: 1000 µm

Figure 39 presents the retention profiles observed for different microparticles on gastric mucosa through the series of captured fluorescent photomicrographs. It should be noted that the lowest available magnification of 40x was needed to use for evaluating the whole particle mass. According to the analysis using ImageJ software, it is confirmed that every type of microcapsules could remain to some extent on the gastric mucosa even up to 2 h. Among the test particle types, chitosan coated alginate particles exhibited the greatest retention ability, comparable to that of solely chitosan ones. For this formulation, around 62% and 32% of remaining fluorescent intensity could still be observed after 60 min and 120 min of washing, respectively. On the other hand, more rapid removal was observed in case of pure alginate, especially over the last 50 min of the 2 h experiment. Slightly weaker

retention of resistant starch-alginate particles was observed compared to pure alginate microcapsules (Figure 39). Improved mucoadhesion, however, can be feasible via some chemical modifications or addition of specific functional groups (Jelkmann et al., 2019; Kaldybekov et al., 2018). It should be noted that these weak gastric-mucoadhesive characteristics can be preferable if the primary site of therapeutic action of the particular probiotic strain is the intestinal tract. The exemplar series of fluorescent images representing the retention rate of each examined formulation are shown in Figure 40.

Overall, as it can be seen from the present results, the best retention was shown by the chitosan coated alginate particles. This superior retention of chitosan particles could be related to their stronger nature of electrostatic attraction with mucin compared to hydrogen bonds formed by alginate-based particles (Cook et al., 2011; Khutoryanskiy, 2014). Furthermore, the weakest mucoadhesive characteristics was observed for the resistant starch-alginate particles, which could be due to that starch is a non-ionic polymer and these should be typically less adhesive compared to ionic polymers (Khutoryanskiy, 2014). Elzatahry et al. (2009) also reported a satisfactory retention ability of chitosan coated alginate beads on rat intestinal (jejunum) mucosa, with alginate beads formed by extrusion method.

As some recent reports (Bracker & Stender, 2019; Tortajada-genaro et al., 2019) also suggested the potential utilisation of a **low-cost portable USB microscope** in different imaging-related assays, here, this device was attempted to be used for fluorescently imaging the capsule samples and for assessing its usability as an alternative imaging tool for the present mucoadhesive study. As a result, I found that the present retention results conform to previous related findings and expectations (Khutoryanskiy, 2014), and the applied portable microscope (along with the use of an UV light torch) can clearly detect changes in fluorescent intensity needed for the mucosal retention analysis. This, therefore, revealed that the portable USB microscopy device can serve as a potential alternative imaging tool for performing retention studies compared to the conventional fluorescent microscopes. Moreover, the use of the present experimental setup can offer a number of advantages over the traditional fluorescent microscopy method, including the possibility for real-time imaging and detection capability in micro-scale resolution, video recording capability, user-friendliness, portability, increased affordability, and availability of analysis.

4.5 EVALUATION OF THE PROBIOTIC-LOADED CAPSULES PREPARED BY LAYER-BY-LAYER SELF-ASSEMBLY OF POLYELECTROLYTES

4.5.1 Preparation of layer-by-layer assembled polyelectrolyte-based microcapsules

L. casei 01 bacteria were also attempted to encapsulate by alternating coating of them with oppositely charged polyelectrolytes via electrostatic adsorption. In this work, the chosen oppositely charged polyelectrolytes were carboxymethyl cellulose (–) (CMC) and chitosan (+) (CHI). Contrary to the previous encapsulation techniques, this one rather involved a coating process of individual bacterial cells, instead of embedding multiple cells within a polymer matrix(ces). Because of it nano-sized capsule walls (layer structure) are expected to be formed with this encapsulation approach. In this work, a total 6 alternating depositions of these polyelectrolytes were applied on the cell surface, in the following order: CMC (–) / CHI (+) / CMC (–) / CHI (+) / CMC (–) / CHI (+) (i.e. 3x CMC / CHI bilayers). Starting off with CMC coating was necessary because when the first incubation (i.e. coating) of cells was conducted in CHI solution, the subsequent removal of this polyelectrolyte solution (i.e. cell pellet formation) with centrifugation could not be possible; this failure consequently made it difficult to carry on with the coating process. Besides, CHI has been associated with its possible broad-spectrum antibacterial effects (No et al., 2002), because of which the direct contact of bacteria with this polymer may have been problematic. After the whole coating process, these formulations were also evaluated in the following physical and physiological aspects.

4.5.2 Microscopic evaluation of *L. casei* 01 following the multicoating process with bilayer of carboxymethyl cellulose and chitosan

For detecting the possible changes on (or around) the bacterial surface after the coating process with alternate depositions of CMC and CHI, inverted microscopy observation at 100x magnification was applied along with fluorescence or/and phase contrast modules. Figure 41A, 41B and 41C show different variations of the microscopic images of these bacteria. In Figure 41A, which was acquired by overlapping the phase contrast and the fluorescence images, a slight autofluorescence could be clearly detected on and around the bacterial cells, especially within the aggregated mass. The enhanced autofluorescence within these particular areas was even more noticeable on the image captured under fluorescence (Figure 41B).

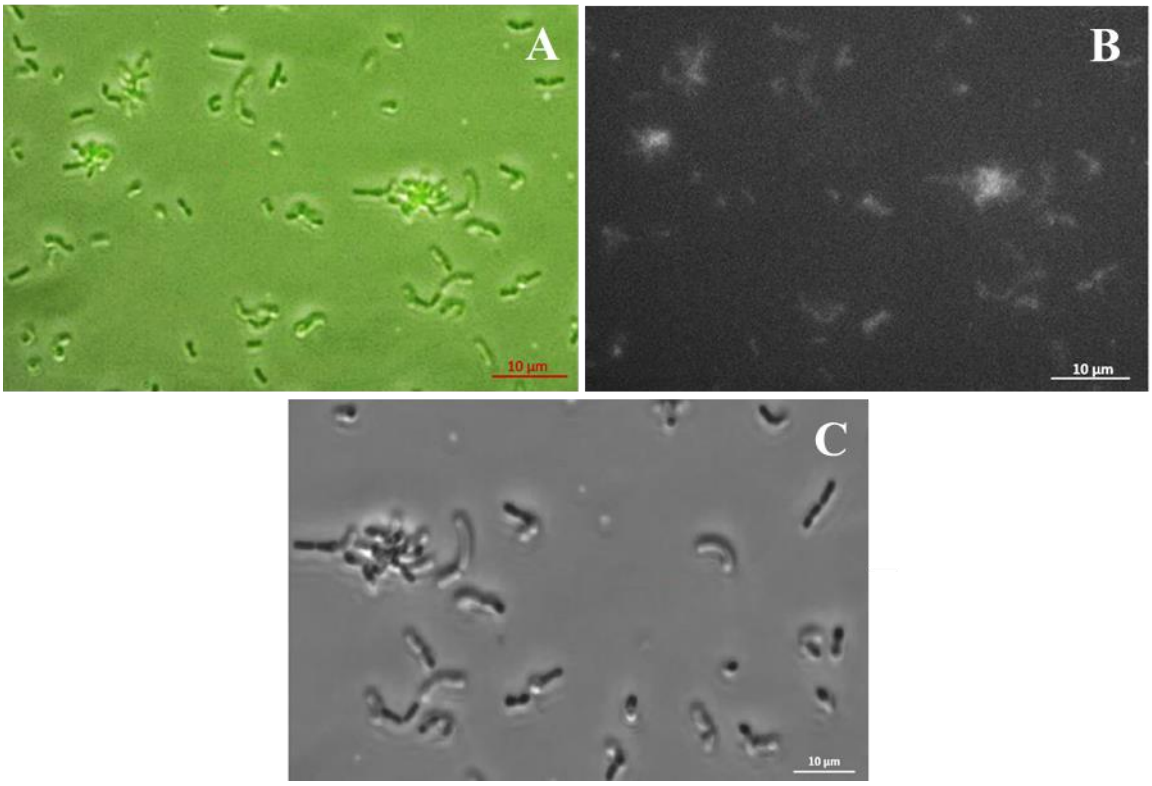


Figure 41. Inverted microscopy images of *L. casei* 01 cells after muitiple, alternate coating with CMC / CHI: overlapping fluorescence and phase contrast (A), grayscale fluorescence (B) and phase contrast (C) images. Applied optical magnification: 100x. Scale bar: 10 µm

By observing with the phase contrast imaging technique, some bacterial cells with distinctive brighter ('whitish' grey) surface could be seen (Figure 41C), which may be caused by the presence of the polyelectrolyte layers on the bacteria. However, more research is necessary to find out the actual reason and explanation for this observation. With this microscopical analysis, no modification was noticed regarding the morphology of the bacteria after the coating process.

4.5.3 The effect of the number of polyelectrolyte coating application on the physiological activities of *L. casei* 01 bacteria

L. casei 01 bacteria were regrown and enumerated in MRS agar (as described in Section 3.3.2) after each alternating coating step with either CMC or CHI, to find out the effect of each number of layer deposition on the physiological activity of these bacteria and, at the same time, to determine the successfulness of this encapsulation approach. The enumerations of these bacteria were always done after the same incubation time in MRS agar. Furthermore, different initial bacteria concentrations (to be coated) were applied to gain some additional knowledge regarding the coating yield with this

coating procedure. To do this, two types of multiple CMC/CHI coated bacterial formulations were prepared from two different volumes. The two different initial viable cell concentrations of this bacterial suspension were 11 log CFU/mL and 10 log CFU/mL. For the better comparison, these results are presented in relative percentage values in Figure 42.

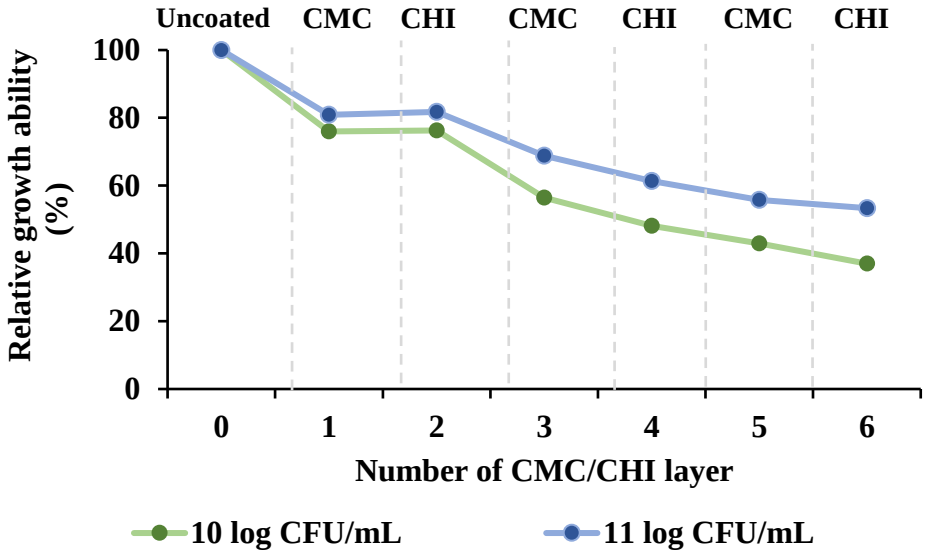


Figure 42. Growth ability of *L. casei* 01 after each step of alternating coating with either carboxymethyl cellulose (CMC) or chitosan (CHI), and its dependent on their concentration to be coated

According to the obtained data, viable counts of *L. casei* 01 showed a general decreasing tendency with each additional polyelectrolyte layer application. However, they were still capable to grow even after coating with the 6th (last) polyelectrolyte layer. The possible explanations for this observation can be that each of these layers was successfully deposited on the bacterial surface, by which available nutrients could be increasingly blocked from being utilised immediately; this inhibition likely prolonged the lag phase of growth cycle (Priya et al., 2011), which eventually induced delayed (slower) growth mechanisms for the bacteria. In this regard, no difference was observed as to which concentration (volume) of bacteria suspension was applied, however, it rather affected the decrease rate. Decrease rate was generally found greater when lower concentration of bacteria (10 log CFU/mL) were coated. This may indicate that some amount of the bacteria remained uncoated or not coated equally with all the layers, supposing that higher yields of coating can be obtained for less concentrated bacteria suspensions (with fixed concentration of polyelectrolyte). Accordingly, higher

yield of coated bacteria could lead to less remaining free bacteria that could still exhibit normal reproduction. With regard to the first 4 layers, it was also apparent that more significant reductions occurred typically after the deposition of the CMC layer. While reductions of around 21 % and 16 % were observed with the 1st and 3rd coating with CMC, the 2nd and 4th coating with CHI brought about only 0.5 % and 7.5 % reductions in the bacterial growth, respectively.

The smaller decrease of growth rate observed after each CHI-based coating might be related to the low molecular weight of this polymer, by which the impermeability of the whole assembled polyelectrolyte multilayer could not increase as much as with CMC-based coating. With the additional 5th and 6th layer depositions, the decrease tendency of viable counts was more gradual, reflecting that the adsorption of these last two coatings could be somehow less feasible than that of the previous ones. By expressing with CFU values, a final growth of around 6 log CFU/mL was detected (after the 6th layer application) in case of the greater initial bacterial concentration (11 log CFU/mL, 1 mL), while the bacteria ended up with a growth of around 4 log CFU/mL in case of the smaller initial bacterial concentration (10 log CFU/mL, 0.1 mL).

In overall, based on these obtained results, the applied coating process itself was revealed as effective or at least until the 4th coating application, and the coated bacteria could still remain viable. However, increasing number of adsorbed coating layers could come with decreasing permeability to available nutrients, thereby decreasing (delayed) physiological activities of the coated bacteria. This agrees with the finding reported by Priya et al. (2011).

4.5.4 Viability of multiple layer-by-layer CMC/CHI coated and uncoated *L. casei* 01 in simulated gastric and intestinal conditions

To investigate the effect of multiple layer coatings on the tolerance of *L. casei* 01 for strong acidic and bile salt conditions, survival studies were carried out separately with simulated gastric fluid (SGF) (including pepsin) and simulated intestinal fluid (SIF). Uncoated (free) bacteria were also examined as a control sample. Bacteria were exposed to these stress conditions after coating them with 4 coating layers (CMC/CHI/CMC/CHI). The results (Figure 43) obtained from these tolerance tests showed that the multiple CMC/CHI coatings could somewhat enhance the viability of *L. casei* 01 bacteria while exposed to either SGF or SIF, which can be related to the decreased permeability provided by this multiple polyelectrolyte barrier formed on the bacteria surface. Another possible explanation for this is that chitosan – as the last applied coating layer – may exert its acid buffering effects aroused from its cationic nature (Cook et al., 2012).

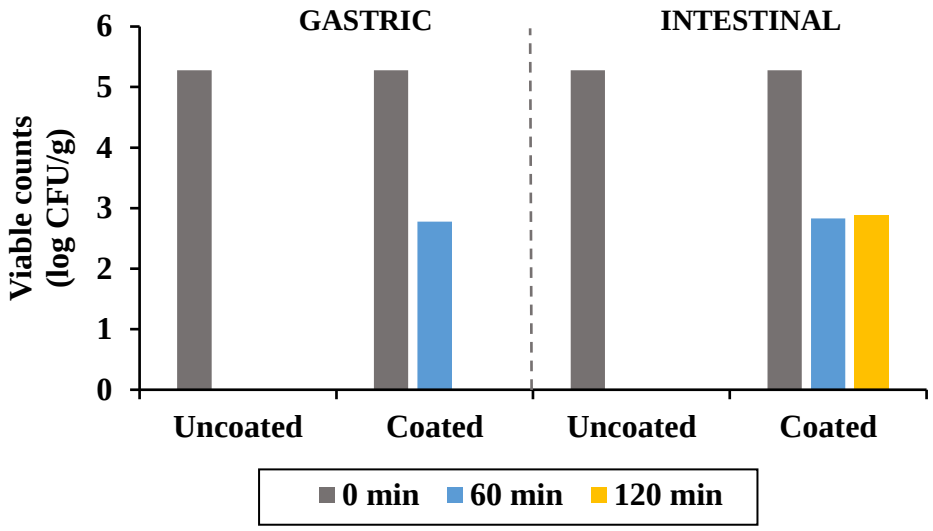


Figure 43. Viability of uncoated and layer-by layer (2 x CMC/CHI bilayer-) coated *L. casei* 01 during separate exposures to simulated gastric and intestinal fluid

Unlike the uncoated cells, some viable counts were still maintained with these coatings after 1 h of exposure to SGF, with the average loss of 2.7 log CFU/g. However, within an additional hour of SGF treatment, viable cell counts decreased to undetectable level, with a total loss of minimum log 5 CFU/g. This result indicates that such acidic conditions can be still lethal even to the coated bacteria, when exposed for a certain long time (> ~ 1 h). This coating appeared to provide better bacteria protection against longer exposure in bile salt-contained conditions, in a way that a total viability loss of only 2 logs was observed by the end of 2-h exposure (Figure 43). Interestingly, the 2nd hour of SIF treatment did not result any decrease in the bacteria viability.

Priya et al. (2011) with their similar study showed that *L. acidophilus* with CHI/CMC/CHI (3 layer) coating managed to survive the simulated gastric conditions with an only viability loss of 0.8 log even after 2-h period, outperforming that obtained for *L. casei* 01 in my study. Although Priya et al. did not assess their acidic (gastric) tolerance in uncoated form, this can possibly be attributed to the generally good acid tolerance of *L. acidophilus* demonstrated in other previous study (Soliman et al., 2015). Better survival during 2-h of gastric incubation than obtained in this study was also achieved when *Bacillus coagulans* were coated with single and double bilayers of CHI/ALG and two bilayers of CHI/ Eudragit (L100). However, when exposed to bile solution for 2 h, only the two bilayers of CHI/ALG provided a satisfactory protection and the other examined coated formulations mentioned above performed even weaker than the ones applied in my work (Anselmo et al., 2016).

If comparing this encapsulation approach with the previous ones in this work, the extrusion-based encapsulation provided partly better protection for *L. casei* 01 under the same SGF conditions (either with or without pepsin activity), fairly depending on the applied encapsulating materials (Section 4.2.6). In the first roughly one hour of treatment, the viability was mostly better (or at least equally) protected with extrusion-based encapsulation. Afterwards, better viability protections even up to around 2 h of exposure were only observed when bacteria were (extrusion-based) encapsulated in prebiotic-contained alginate matrices, such as resistant starch-alginate, lactosucrose LS40L-alginate, lactosucrose LS55L-alginate, and in the chitosan coated alginate capsules. Similar survival profiles as the present ones were seen with emulsification-based encapsulation, by which resistant starch-alginate capsules could also protect *L. casei* 01 well under the same bile salt-contained SIF conditions, but also failed to do so under gastric conditions (Section 4.3.4). With respect to *L. plantarum* encapsulated in electrosprayed microcapsules, they also showed similar rate of viability loss after 1 h and 2 h exposure to SGF when encapsulated in alginate capsules. However, it was improved a lot with resistant starch blending and chitosan coating (Section 4.4.4).

5 CONCLUSIONS AND RECOMMENDATIONS

In my research work, I evaluated different ***encapsulation materials*** and ***encapsulation techniques*** for their suitability to develop effective (micro)capsules as protective and gastrointestinal (targeted) delivery systems for probiotics. To this end, various **physical** and **physiological evaluations** were performed on the resultant capsule formulations.

Ionotropic gelation approach like ***extrusion technology*** has been revealed to be suitable for encapsulating probiotic *Lactobacillus casei* 01, using food grade hydrogel biopolymers like ***2 % (w/v) sodium alginate***, and different blends of ***0.75% (w/v) gellan gum - 1% (w/v) xanthan gum*** and ***2 % (w/v) κ-carrageenan - 1% (w/v) locust bean gum***. The resultant physical and functional (protective) characteristics of these capsules are highly dependent on the encapsulating materials. First, **spherical-shaped** capsules can be formed with sodium alginate, whereas rather irregular-shaped capsules can be formed with the non-alginate biopolymers. Combination with other carbohydrates, specifically prebiotics differently modifies the shape of alginate capsules depending on the specific type; for instances, ***2 % (w/v) lactosucrose*** (both ***LS40L*** and ***LS55L***) and ***2 % (w/v) lactulose*** seem to decrease the sphericity of the formed alginate capsules by apparently lowering the viscosity of the alginate solution, whereas ***2 % (w/v) resistant starch*** does not modify (or even improves) that. Spherical shape of alginate capsules can be also unchanged in the case of applying polymer coating layer like ***chitosan*** or ***DEAE Sephadex A 50***. Furthermore, **capsule size** is also influenced by the used building materials as smaller sized capsules can be formed with gellan gum – xanthan gum, but greater sized capsules can be resulted with κ-carrageenan - locust bean gum, compared to alginate capsules. The size of these alginate capsules seems to be slightly increased by blending with specific carbohydrates (i.e. resistant starch, lactosucrose) or by coating with specific polymers (i.e. chitosan). As is obvious, capsules with smaller size range would be more preferable when it comes to incorporating them in commercial food products. The data obtained from texture analysis indicate that sodium alginate gives better overall **mechanical characteristics and physical stability** to the resultant capsules than κ-carrageenan - locust bean gum and especially gellan gum – xanthan gum; in fact, this can be additionally improved by blending with prebiotic lactulose, lactosucrose LS55L and resistant starch, whereas alginate gel matrix tends to drastically lose its firmness by the coating process with chitosan. Interestingly, the non-alginate biopolymers (i.e. gellan gum-xanthan gum, κ-carrageenan-locust bean gum) seem to be better choices than alginate when it is about the **encapsulation yield** of viable cells. Although, it can be significantly improved by incorporation of prebiotic component or by applying chitosan coating.

When it comes to protecting the probiotic viability against environmental stress factors, it can be demonstrated that the inclusion of prebiotic excipient, especially resistant starch in alginate matrix affects most positively the survivability of probiotics under **strong acidic (pH = 2)** and **bile salt conditions** (typical of the gastric and intestinal environment), compared to other examined capsule variations (i.e. pure alginate, two coated alginate and two non-alginate capsules).

In fact, it has also been found – by applying a more sophisticated **Infogest** *in vitro* digestion protocol – that the starch-contained capsules would also highly withstand a more complex digestion conditions regarding the ionic strength and enzymatic activity (α-amylase, pepsin, pancreatin), which closer represent the *in vivo* human physiology. With respect to the survival rate in simulated gastric fluid, it has been also revealed that **pepsin** has significant effect on the probiotic protection ability of the capsules as it can be decreased without the presence of that enzymatic activity in stomach (e.g. during the fasted state). In the case of **long-term storage in plant-based beverages like (pre-fermented, acidic) oat and beetroot drinks**, this resistant starch-alginate -based encapsulation has been revealed to improve the shelf-life of (free) *L. casei* 01. Although, this stability improvement seems to be only relevant when storage lasts for minimum 3 months since probiotics even without the use of encapsulation can survive comparably well in the first 2 months of storage. This survival (protection) profile can also depend on the type of plant-based matrices. For example, in the case of storage in cereal-type beverage like oat drink, the actual protection effect of this encapsulation can be only expected at ambient temperature (20°C), whereas, in the case of vegetable-type beverage like beetroot drink, this encapsulation tends to improve the storage stability of probiotics not only at ambient (20°C) but also at refrigeration temperature (4°C). The acidification kinetics of encapsulated probiotics showed that only minimal production of lactic acid can occur during 5 months of storage in these plant-based beverages, and even so, mainly over the 1st month period and under ambient (20°C) conditions. However, more remarkable increase can be expected in the butyric acid content of beetroot drink especially in the case of the 1st month of ambient storage [by about 0.6 % to 1.7 % (w/v)]. Based on this result, the greater sensitivity of free *L. casei* 01 to the storage in beetroot drink and/or under ambient conditions may be attributed to the greater acid content resulted in these cases – which has been shown to be improved by encapsulation. Furthermore, the acidification rate has been also shown to not differ considerably between the free and encapsulated probiotics-contained drinks.

It has also been found that the protective effect of encapsulation can vary at genus-specific level as it exhibited to be greater on the viability of *Bifidobacterium lactis* Bb-12 than *Lactobacillus casei* 01 under conditions such as heat, strong acid and bile salt. Especially, in the case of **heat treatment** at

60°C, extrusion-based encapsulation did not provide much better heat stability to the lactobacilli than that of free cultures, but it clearly improved that (by around 15 min) to the bifidobacteria one.

As opposed to extrusion, smaller sized capsules (roughly in the range of 0.8 - 10 mm) can be partially formed by ***emulsification/external gelation*** method, which would be generally more favourable for food incorporation. However, it has been revealed that encapsulation by this technique – into the same resistant starch-alginate gel matrix – tends to provide generally worse protection of probiotics from the challenges of strong acid and the high temperature than when encapsulation is done by extrusion technique. Although, in practical aspect, the long-term storage stability of probiotics in low pH of plant-based matrices seems to be rather irrelevant as to which of the two encapsulation procedures is applied.

By ***electrospray***ing of the same resistant starch-alginate, it is possible to encapsulate probiotics into capsules with both uniformly fine size – in the range of 30-600 μm – and spherical shape, unlike with the extrusion or the emulsification techniques. It further appears that, encapsulation into these fine sized capsules can still result in high encapsulation yield and high protection of probiotics against strong acid challenge during the simulated gastric treatment. By comparing the observed protection ability of this electrosprayed microcapsules with that of the much greater sized capsules formed by extrusion-based method, this study has not proved the earlier hypothesis (Argin, 2007; Chandramouli et al., 2004; Cook et al., 2012; Lee & Heo, 2000; Muthukumarasamy et al., 2006) suggesting that the protection of encapsulated probiotics under acidic conditions is enhanced with increased capsule size. Although, this finding may not be concluded fully from this comparison since two different species of *Lactobacillus* were applied and examined in the extrusion (*L. casei*) and electrospray (*L. plantarum*) encapsulation studies.

Furthermore, I also found that although blending with resistant starch has the great protective effect, the **mucoadhesive property** of alginate capsules – which is important for assuring the extended residence time of encapsulated probiotics within the gastrointestinal tract for exerting their health effects – tends to be weakened by the effect of the non-ionic nature of starch, although not by a great extent. This property, on the other hand, can be considerably improved if capsules are supplied with cationic chitosan coating, through which strong ionic interaction can potentially be established with anionic mucin.

Finally, apart from the previously mentioned methods, microencapsulation of probiotics can be also achieved by individually coating individual cells through ***layer-by-layer (alternating) self-assembly*** of two oppositely charged polyelectrolytes like carboxymethyl cellulose (–) and chitosan (+) (in this

order). However, this coating approach tends to limit the physiological activities of probiotic cells as more and more layers are assembled on their surface; for example, after coating with 4-6 polyelectrolyte layers, around 50% recovery rate of bacteria can be only obtained on the same growth medium. Furthermore, this encapsulation approach with 4 alternate layers can fall short in the protection of probiotics under strong acidic (pH = 2) and bile salt conditions compared to extrusion-based encapsulation with resistant starch-alginate or other prebiotic-blended alginate matrices, or electrospray-based encapsulation with resistant starch-alginate.

Overall, I demonstrated from my results that the use of ***electrospray*** as encapsulation technique and prebiotics, especially ***resistant starch*** as encapsulants (excipients) can play a potential role in the development of effective and ideal micro delivery systems for probiotics that can maintain their viability above the minimal recommended level of 6 log CFU/g or mL (Yao et al., 2020) during their delivery and also can be put forward for plant-based food applications, thereby offering the possibility to develop novel probiotic non-dairy food products. It is also worth mentioning that electrospray-based encapsulation would be also easily adaptable for industrial-scale applications and, without the necessary use of intensive heating, it can be also more cost-effective than the similar but more commonly reported encapsulation techniques such as spray drying. However, with a view to design a proper controlled gastrointestinal delivery of encapsulated probiotics, the weak mucoadhesive performance arising from the inclusion of non-ionic components like starch still needs to be overcome. For this purpose, one of the potential approaches may be to apply chitosan – or other highly mucoadhesive polymer – coating in combination with the prebiotic (resistant starch)-blending. Furthermore, there is still room for improvement in terms of probiotic protection at high temperature conditions in order to safely expose them to such common technological processes like pasteurisation or sterilisation (if needed for the specific applied probiotics and the technological processing of foods). Finally, it is also recommended that further research be undertaken in the following aspects and areas (among others): (1) assessing the release nature of probiotics from these starch-contained microcapsules to the target site within gastrointestinal tract as the percentage of the probiotics delivered may be considerably less than that administered; (2) further assessing the protection ability of the microcapsules using *in vivo* approaches or at least a dynamic *in vitro* gastrointestinal model system (e.g., SHIME, TNO), considering that the real physiology of human gastrointestinal tract is highly complex and tends to vary greatly between subjects and several factors such as time since eating and age (Cook et al., 2012); (3) further studying the effect of lactosucrose LS55L and lactulose,

or even other types of prebiotics as encapsulants on the characteristics and protection ability of microcapsules.

6 NEW SCIENTIFIC RESULTS

1.) I proved that the physical stability (regarding hardness and springiness) of the resultant alginate gel capsules was greatly increased by the addition of 2% (w/v) resistant starch into alginate, and I also demonstrated that the probiotic protection effect of alginate capsules against simulated gastric and intestinal conditions was also substantially improved with this firmer textural characteristic. Moreover, investigating with Infogest gastrointestinal model system, the protective effect of blend resistant starch-alginate capsules was highly satisfactory in a way that they could maintain the viability of probiotics above the recommended minimum level of 6 log CFU/mL for realising the therapeutic effects thereof.

2.) I found that with the encapsulation technology, especially with the resistant starch-contained capsules, the long-term storage viability of probiotic *L. casei* 01 could effectively maintained above the recommended minimum level of 6 log CFU/mL not only through refrigerated storage at 4°C, but even through 3 months of storage at 20°C in either oat or beetroot drinks. This result advocates a great promise of developing an effective probiotic plant-based products, considering that many of the commercial plant-based food products are rather stored and marketed at room temperature.

3.) By examining different encapsulation techniques, I proved that electrospray technique was the most promising approach to microencapsulate probiotics as applying this technique not only ensured high encapsulation efficiency (~ 87 %) and high viability protection (with an only loss of 3.68 log CFU/mL through 2h of simulated gastric treatment), but also enabled a production of the capsules in micron-size range (30 – 600 μm). Formation of microcapsules in this size range would enhance the adaptation of this type of microencapsulation into food industrial applications and would also allow a more cost-effective mass industrial production of the microcapsules.

4.) I found that same encapsulation (i.e., extrusion and emulsification) approaches provided better viability protection for *Bifidobacterium lactis* Bb-12 against the same strong acidic and bile salt effects of *in vitro* gastrointestinal conditions and particularly against the high-temperature (60°C) conditions, as compared to that for *Lactobacillus casei* 01. With this, I proved that the effectiveness of encapsulation can vary at a strain-specific level.

5.) With a portable microscope (1080P 1000X Zoom HD 8LED Digital USB Microscope Magnifier Endoscope Video Camera) and an UV torch, I constructed and tested an experimental set-up that can be used as an effective alternative approach to performing a

fluorescence imaging-based retention test for mucoadhesion analysis. With this approach of experiment, there are a number of technical and economic advantages offered to researchers over the traditional fluorescent microscopy approach (e.g., the possibility for real-time monitoring and imaging the formulations in micro-scale resolution, the possibility for video recording the whole *in vitro* wash-off process, the user-friendliness and increased affordability of the experiment).

6.) By analysing the retention rate of different alginate-based gel capsules on *ex vivo* porcine gastric mucosa, I observed that the mucoadhesive property of the alginate capsules was only slightly weakened (by about 5.8 % of retention rate) when blending with resistant starch was applied. In this case, a retention rate of around 60 % on *ex vivo* porcine gastric mucosa could be still observed after 50 min of washing with simulated gastric fluid.

7 SUMMARY

Probiotics have a long overall history of human use in several parts of the world for their proposed health-promoting effects. Nowadays, they are principally included in several commercial processed foods as functional ingredients or in nutritional supplements due to increased health awareness among consumers. However, the potential health benefits of probiotics – thereby the probiotic products – may not be realised because of the possibility of their drastic viability and functional losses during the food processing (e.g. heat treatment), long-term household storage (e.g. high acidity of food matrix) and the subsequent gastrointestinal transit (e.g. passage through strong acidic gastric fluid). Microencapsulation as one of the most modern approach can potentially be used to enhance the resistance of probiotics to various environmental stresses and effectively deliver them to their therapeutic sites of action within the human gastrointestinal tract (e.g. colon). So far, a wide range of gastrointestinal delivery (capsule) systems for probiotics has been developed in different previous studies. However, there are still many shortcomings that limit the adaptation of these systems for food industrial and commercial applications. These include the high production cost, too large capsule size that can negatively affect the sensory traits (e.g., texture, flavour) of food products, low scalability, insufficient cell protection ability, or that the ingredients or polymer wall/matrix used for the encapsulation is not suitable for food applications, among others (Yao et al., 2020).

In light of above-mentioned concerns, my PhD research work was designed to find the most suitable method and encapsulation material for development of microcapsules that can be utilised for effective protection and gastrointestinal delivery of probiotics, and at the same time, can be well-adapted for food industrial and commercial applications, thereby for developing novel probiotic non-dairy, particularly plant-based food products. To this end, *Lactobacillus casei* 01 (as a model probiotic strain) was first encapsulated by extrusion technique into different matrix variations of the capsules including calcium alginate [2 % (w/v)], calcium alginate blended with prebiotics like resistant starch, lactosucrose LS40L, lactosucrose LS55L and lactulose [2 %-2 % (w/v)]; and non-alginate blends like gellan gum – xanthan gum [0.75 % -1 % (w/v)] and κ-carrageenan-locust bean gum [2%-1 % (w/v)]; and these gel capsules were then evaluated for their physical properties (size, shape, texture), encapsulation efficiency, and bacterial protective performance under *in vitro* digestion conditions. Furthermore, the effect of polymer coating of calcium alginate capsules with either chitosan or DEAE Sephadex was also evaluated in the same aspects as above.

Results showed that the physical (size, shape, texture) and physiological characteristics (encapsulation efficiency, bacterial protection ability) of the capsules produced varied with the type of applied

encapsulating materials. For utilising as probiotic delivery systems, I revealed that those alginate capsules that blended with prebiotics had the most ideal characteristic since these simultaneously had the firmest gel structures, provided the highest encapsulation yields (ranged between 77 % – 79 %) and the most effective protection of probiotic viability, especially the resistant starch-blended alginate ones, under strong acidic (pH = 2, either without or with pepsin activity) and bile salt conditions of simulated gastrointestinal fluids. In fact, I also reported a highly effective protection of probiotic viability with resistant starch-alginate capsules when examined with a more sophisticated, standardised static *in vitro* digestion protocol of COST Infogest network (Minekus et al., 2014); in this case, probiotic viability was only lost by roughly 1 log CFU/g after the sequential phases of simulated oral (2 min) (with amylase activity), gastric (120 min) (with pepsin activity, pH = 3) and intestinal (with bile salt content and pancreatin activity) (120 min) conditions. By further evaluating these resistant starch-blended alginate capsules, I observed that this encapsulation increased the long-term storage stability of the probiotics in acidic plant-based beverages like pre-fermented oat- and beetroot drinks, to the extent of maintaining the probiotic viability above the suggested minimal viable cell counts (Yao et al., 2020) even without the refrigerated storage and even for 3-4 months Although, the positive effect of encapsulation was only clearly seen when the storage was performed for at least 3 months as the unencapsulated cells could also survive comparably well in the first 2 months' period. The effect of different storage temperature on the bacteria stability varied with the type of plant-based matrices; more specifically, the storage stability improvement in oat drink was only observed at ambient (20 °C) temperature, whereas the stability in beetroot drink was clearly improved under both ambient (20 °C) and refrigerated (4°C) storage conditions. According to the heat-treatment experiment performed in this work, the resistant starch-alginate encapsulation appeared to only slightly improve the thermal stability of (free) *L. casei* 01 at 60°C, and it failed to do so at a higher, 85°C temperature.

Considering that extrusion technique resulted unfavourably large capsule size (~ 2 – 5 mm) for food incorporation, I carried out further probiotic encapsulation studies with emulsification/external gelation and electrospray procedures using the same resistant starch-alginate (shortlisted as a encapsulation material candidate for providing the greatest probiotic protection in the previous extrusion-related study), and compared them with the extrusion technique in terms of suitability to formulate proper probiotic delivery systems. This comparison showed that all these applied techniques were suitable for encapsulation of *L. casei* 01 with high yields, however, uniformly fine spherical capsules (in size range of even 30-600 µm) could be only obtained by electrospraying approach, with which probiotic viability could still be highly protected under stress conditions such as strong acidic

simulated gastric fluid (pH = 2, without pepsin), comparable to that with the extrusion-formed ones. In fact, I obtained generally weaker protection of probiotics with emulsification-based encapsulation than with the extrusion one.

Furthermore, I also demonstrated that blending with resistant starch weakened the retention of alginate capsules on an *ex vivo* porcine gastric mucosa. However, the use of chitosan coating considerably improved the mucoadhesive characteristics of alginate capsules. Besides good viability protection, good mucoadhesion of the capsules has also been reported to be important for extending the residence time of encapsulated probiotics within the gastrointestinal tract and thereby for better controlling their gastrointestinal delivery (i.e. better exerting the probiotic-based gastrointestinal therapy) (Khutoryanskiy, 2014).

By investigating another probiotic bacteria of different genus, extrusion and emulsification-based encapsulation could also successfully be applied to *Bifidobacterium lactis* Bb-12, in which case higher level of viability protections were generally observed under the same challenges of *in vitro* gastrointestinal and especially high temperature (60°C) stresses. This result indicates that the effect of encapsulation can vary in this regard.

As a fourth type of (distinct) encapsulation study, I also encapsulated *L. casei* 01 by electrostatic layer-by-layer (alternating) self-assembly of two oppositely charged polyelectrolytes like carboxymethyl cellulose (–) and chitosan (+). This encapsulation approach differed from the three previous ones in that probiotic cells are individually coated with nano-sized multilayered polymer film, rather than entrapping multiple cells within a polymer (gel) matrix. In my work, total 6 alternate polyelectrolyte layers (carboxymethyl cellulose/chitosan) were shown to be successfully deposited on the bacteria surface. However, I observed that the physiological activity of the bacteria decreased as more and more polyelectrolyte layers were applied on the bacteria surface. As for the protective effect, 4 alternate carboxymethyl cellulose/chitosan layers improved the viability of *L. casei* 01 under strong acidic (pH = 2, with including pepsin) and bile salt conditions, but fell short when compared with extrusion-based encapsulation with resistant starch-blended alginate or other prebiotic-blended alginate (among others), or with electrospray-based encapsulation with resistant starch-alginate.

In conclusion, encapsulation of probiotic bacteria in prebiotic-contained, especially resistant starch-contained capsules can be a useful approach to protect probiotics against different environmental stresses and deliver them to gastrointestinal tract in sufficient viable number, with which their health promoting effects and thereby the health claims of probiotic foods can be realised. Furthermore, among the examined encapsulation techniques, electrospray-based encapsulation has the most

potential for food industrial and commercial applications for allowing not only high encapsulation yield and protection level of probiotics but also allowing a capsule formation in a fine (micron) size range that can be well incorporated into food products – as opposed to the ones formed by extrusion. As further benefits, the use of electrospraying also easily enables industrial scale mass production of microcapsules and can be more cost-effective than the other similar, more widespread encapsulation techniques such as spray drying by which intensive heating is principally used. The overall results obtained with my research work can greatly contribute to the design of ideal micro delivery systems of probiotics and thereby to the development of novel and effective probiotic non-dairy, specifically plant-based food products.

In this field, further research should concentrate, among others, on the improvement of thermal protection (if needed for the specific applied probiotics or/and the technological processing of foods) and gastrointestinal mucoadhesive property of resistant starch-based microcapsules in order to develop an even better controlled gastrointestinal delivery of encapsulated probiotics. Besides, further investigations should also be devoted to determining the gastrointestinal release nature of the microcapsules (for ensuring a precise control of the release mechanism of probiotics from the aspects of target site and time point); to determining the protection ability of the microcapsules through *in vivo* or at least dynamic *in vitro* digestion approaches, and also to discovering the possible benefits of other types of prebiotics (aside from resistant starch) or other polymers regarding the proper protection and delivery of probiotics.

ÖSSZEFOGLALÁS

Napjainkban a megfigyelt számos, az emberi egészség számára előnyös tulajdonságuk miatt világszerte egyre jobban elterjed a probiotikumok mint funkcionális élelmiszer-összetevőként vagy táplálékkiegészítőként történő fogyasztása. Azonban számos irodalom leírta, hogy a probiotikumok – és ezáltal a probiotikus termékek – egészségügyi hatása megkérdőjelezhető, ugyanis az élelmiszer-feldolgozás (pl. a hőkezelés), a hosszú távú tárolás alatt az adott élelmiszer közegben (pl. erős sav közegben) és az azt követő emésztési (gyomor-bél rendszerben való átjutási) folyamat során ért stresszhatások miatt drasztikusan lecsökkenhet a probiotikumok életképessége és ezáltal a funkcionális képessége is. Mikrokapszulázási technológia alkalmazása – vagy más szóval probiotiotikumot hordozó rendszer (mikrokapszula) fejlesztése –potenciális megoldást jelenthet arra, hogy a probiotikumok különböző környezeti stresszhatásokkal szembeni ellenálló képességét fokozzuk és ezáltal megfelelő életképességgel eljutassuk ezeket az emésztő rendszerbe (pl. vastagbélbe). Ezidáig korábbi tanulmányokban számos különböző hatékony mikrokapszulázási technikákat fejlesztettek ki, viszont ezekkel való kapszulázás élelmiszeripari (nagyléptékű) kivitelezhetősége még nem kiforott, többek között a kapszulák magas előállítási költsége, a túl nagy kapszulaméret ami ronthatja az élelmiszertermékek érzékszervi tulajdonságait (pl. állomány, íz), a kapszula elégtelen probiotikum-védő képessége, vagy az hogy a probiotikumok kapszulázásához felhasznált segéd vagy kapszula anyagok nem alkalmasak az élelmiszeriparú célra (Yao et al., 2020).

Mindezek fényében a PhD kutatási munkám célja az volt, hogy megtaláljam a legmegfelelőbb módszert és kapszulázó anyagot olyan mikrokapszulák kifejlesztésére amelyek biztosíthatják a probiotikumok hatékony védelmét és béltraktusba történő hordozását, ugyanakkor élelmiszeripari és fogyasztói célra is alkalmasak, és mindezek által lehetővé tegyék egy új, különösen növényi alapú probiotikus élelmiszerek kifejlesztését. Ebből kifyolólag a *Lactobacillus casei* 01-et (mint modell probiotikus törzset) legelőször az extrúziós technikával kapszuláztam különböző anyagú gélkapszulákba, beleértve a kalcium-alginátot [2 % (m/V)], a kalcium-alginát és valamilyen prebiotikum keveréket, például rezisztens keményítővel, laktoszukróz LS40L, laktoszukróz LS55L és laktulózzal [2 % - 2 % (m/V)] keverve; és nem-alginát alapúakat, például gellán gumi – xantán gumi [0,75 % -1 % (m/V)] és a κ-karragenát – szentjánoskenyér gumi [2 % - 1 % (m/V)] alapút. Ezeket a gélkapszulákat majd megvizsgáltam fizikai tulajdonságaik (pl. méret, alak, állomány), probiotikum-kapszulázási hatékonyságuk, és probiotikum-védő hatásuk szempontjából *in vitro* emésztési körülmények között. Továbbá a kalcium-alginát kapszulák kitozánnal, illetve DEAE Sephadex-szel történő polimer-bevonássának hatását is vizsgáltam ugyanazon szempontok szerint.

Az eredményeim azt mutatták, hogy az előállított kapszulák fizikai (méret, alak, állomány), illetve probiotikum-kapszulázási és -védő képessége nagyban függött az alkalmazott kapszulázó anyagok típusától. Emellett kimutattam azt is, hogy a probiotikumokat hordozó rendszerként történő alkalmazáshoz a prebiotikumokkal kevert alginát kapszulák, különösen a rezisztens keményítővel kevert alginát kapszulák rendelkeztek optimális tulajdonságokkal, ugyanis ezen alapú kapszulák gélszerkezetét mértem a legkeményebbnek, illetve ezekkel értem el a legmagasabb kapszulázási hozamot (77 % - 79 % között), valamint a leghatékonyabb probiotikum-védelmet az *in vitro* gyomor (pH 2, pepszin hatással vagy anélkül is) és epesavas közegével szemben. Ezenfelül, a rezisztens keményítő-alginát alapú kapszulák esetében rendkívül nagy mértékű probiotikum-védelmet tapasztaltam az össztetettebb Infogest standardizált emésztési modell rendszeren (Minekus et al, 2014); ebben az esetben csupán 1 log TKE/g nagyságrenddel csökkent a *L. casei* 01 élő sejtkoncentrációja az egymást követő szimulált szájüregi (2 perc) (amiláz hatással), gyomor (120 perc) (pH 3, pepszin hatással) és vékonybél (120 perc) (epesó és pankreatin hatással) közegben történő kezelés végére. A kevert rezisztens keményítő-alginát alapú kapszuláknál azt is megfigyeltem, hogy ezen típusú kapszulázás jelentősen növelte a *L. casei* 01 hosszú távú életképességét savanyított (fermentált) növényi alapú ital termékekben mint a zab- és céklaital, olyan mértékben, hogy hűtőtárolás nélkül is meg tudta őrizni a terápiás hatás érvényesüléséhez javasolt minimális élő sejtszámot 3-4 hónapon keresztül is (Yao et al., 2020). Fontos lehet megemlíteni azonban, hogy probiotikum-kapszulázás ezen pozitív hatását csak akkor tapasztaltam, ha a tárolást legalább 3 hónapnál tovább végeztem, mivel az első 2 hónapos tárolás során a kapszulázatlan sejtek életképessége is viszonylag magasan megmaradt. A különböző tárolási hőmérséklet hatása a baktériumok eltarthatóságára a növényi alapú termék típusától függően változott, ugyanis zabitalban tárolva csak 20 °C alatt javult a baktériumok életképessége kapszulázással a nem kapszulázott sejtekéhez képest, miközben a céklalé, esetében nemcsak 20°C-on, hanem 4°C-on tárolva is megfigyelhető volt jelentős életképesség javulás a kapszulázásnak köszönhetően. Szimulált emésztés és élelmiszer-mátrixban történő tárolás után, hőkezelés hatását is megvizsgáltam a *L. casei* 01 életképességére, melynek eredményeként azt kaptam, hogy a 60°C-os hőkezelés ellen a rezisztens keményítő-alginát alapú kapszulázás már nem biztosított kiemelkedő védelmet a probiotikumok számára. , nem beszélve a 85°C-os hőkezelés ellen.

Tekintettel arra, hogy a fent említett extrúziós technikával formált kapszulák mérete túlzottan nagy (~ 2 - 5 mm) ahhoz, hogy fogyasztói célú élelmiszertermékbe bevihetőek legyenek, a *L. casei* 01 kapszulázását elvégeztem és tanulmányoztam az externális gélképzésen alapuló emulziós és az

elektrosztatikus porlasztásos (electrospraying) technikával is, a fentiek alapján legígéretesebbnek bizonyuló kevert rezisztens keményítő-alginát felhasználásával. Mind a három technikákat összehasonlítottam egymással a probiotikumokat tartalmazó kapszulák képzése szempontjából, amely azt mutatta, hogy az összes alkalmazott technika alkalmas volt a *L. casei* 01 nagy hozamú kapszulázására. Viszont az általános fogyasztói célra ideális nagyon pici szemcse méretű és gömbszerű kapszulákat (30-600 µm-es mérettartományban) csak az elektro-porlasztásos módszerrel tudtam elérni, amellyekkel ugyanolyan magas probiotikum-védelmet biztosítottam a stressz hatásokkal (pl. szimulált pH 2-jú gyomor közeggel) szemben, mint az extrúziós technikával képzett kapszulák esetében. Fontos megemlíteni, hogy ezzel szemben az emulziós eljárással képzett kapszulák többnyire gyengébb probiotikum-védelmet tapasztaltam mint az extrúzióssal képzettek esetén.

A megfelelő életképesség-védelem mellett a kapszulák elégséges mukoadhéziós (a nyálkahártyamembránon való tapadási) képessége szintén fontos szempont a kapszulázott probiotikumok gyomor- és béltraktusban való elegendően hosszú tartózkodási idejének biztosításához és ezáltal a gyomor- és béltraktusba történő eljutattásuk hatékonyabb szabályozásához szempontjából is (Khutoryanskiy, 2014). Ezel kapcsolatban kimutattam azt, hogy a rezisztens keményítővel való keverés ugyan csak kis mértékben, de gyengítette az alginát alapú kapszulák mukoadhezív képességét az *ex vivo* sertés gyomor-nyálkahártya felületén. Az alginát kapszulák ezen mukoadhéziós tulajdonságát azonban egyértelműen javította a kitozánnal történő bevonásuk.

L. casei 01 után, az extrúziós és az emulziós technikán alapuló kapszulázást sikeresen elvégeztem egy másik probiotikus törzs, pontosabban *Bifidobacterium lactis* Bb-12 törzzsel is. Ebben az esetben azt tapasztaltam, hogy az utóbbi baktérium számára többnyire magasabb szintű életképesség-védelmet nyújtott a kapszulázás azonos *in vitro* gyomor-bél rendszeri és különösen a magas hőmérsékleti (60°C) stresszhatások ellen. Ez az eredmény pedig azt jelzi, hogy a kapszulázás hatása nemzetség szinten eltérhet.

A kapszulázási tanulmányom során elvégeztem a *L. casei* 01 kapszulázását polielektrolit alapú nanokompozit film rétegenkénti önrendeződéses adszorpciójával is (layer-by-layer self-assembly). Ehhez a kapszulázási eljáráshoz két ellentétes töltésű, pontosabban karboximetil-cellulóz (–) és kitozán (+) polielektrolitot használtam, melynek lévén az adszorpciós lépések elektrosztatikus módon valósultak meg. Ez a kapszulázási módszer abban különbözött az előző három technikától, hogy a probiotikumokat sejtenként vontam be az adott kapszulázó anyagokkal és nem több sejtet kebeleztem be egyszerre egy adott polimer (gél) mátrixba. Munkám során összesen 6 polielektrolit réteget

(karboximetil-cellulóz/kitozán) sikerült felvinnem a baktériumok felületére, melynek kapcsán megfigyeltem, hogy minden egyes rétegfelvitel után a baktériumok fiziológiai aktivitása fokozatosan csökkent. Magát a védőhatást illetően megállapítottam, hogy 2 rétegű karboximetil-cellulóz/kitozán nanofilmmel bevonva számottevően javult a *L. casei* 01 életképessége erős gyomor- (pH = 2, pepszinnel) és epesavas körülmények között, ugyanakkor alulmaradt az extrúziós technikával és az elektro-porlasztással készített rezisztens keményítő-alginát kapszulákkal szemben.

Összességében megállapítható, hogy prebiotikummal, különösen a rezisztens keményítővel kevert alginát gélmátrixba történő kapszulázás ígéretes megoldást jelenthet a probiotikumok különböző környezeti stresszhatásokkal szembeni megfelelő védelme és ezáltal a gyomor- és béltraktusba történő hatékony eljuttatásuk szempontjából. Ennek következtében nagy mértékben fokozható a probiotikumok és probiotikus termékek emberi szervezetre kifejtett egészségügyi hatásai is. Továbbá a vizsgált kapszulázási technikák közül az elektro-porlasztás alapú kapszulázás mutatkozott a legígéretesebbnek élelmiszeripari és kereskedelmi (fogyasztói) célú alkalmazás szempontjából, hiszen nem csak magas hozamú probiotikum-kapszulázást és nagy mértékű probiotikum-védelmet nyújtó kapszulákat eredményezett, hanem lehetővé teszi a kapszulák apróbb, mikron méretű részecske formákban történő előállítását is, amelyek ideálissak lennének az élelmiszeripari termékekbe való belekeveréshez – szemben az extrúziós technikával képzettekkel. További előnyként az elekro-porlasztási technikára vonatkozóan megemlíthető még az is, hogy ez a technika lehetővé teszi a mikrokapszulák költséghatékonyabb ipari tömeggyártását olyan hasonló típusú, de elterjedtebb eljárásokkal szemben mint a nagy hőenergiát igénylő porlasztva szárítás. Ezen elért eredményeim pedig nagyban elősegíthetik egy olyan optimális probiotimukat hordozó rendszer kifejlesztését, amellyel új, ténylegesen probiotikus növényi alapú termékek fejlesztése megvalósítható.

Jövőre nézve, érdemes lehet további kutatást elvégezni többek között a kapszulázott probiotikumok hőkezeléssel szembeni ellenállóképességének növelése (amennyiben az adott élelmiszertermék gyártása és/vagy az adott probiotikum egyéni fiziológiai tulajdonsága ezt szükségessé teszi), illetve a rezisztens keményítő alapú mikrokapszulák gyomor és bél rendszeri mukoadhéziós képességének fejlesztése érdekében, hogy ezáltal is hatékonyabb legyen a hordozott probiotikumok gyomor- és béltraktusba történő eljuttatása. Továbbá azon célból, hogy lehetővé tegyük a probiotikumok mikrokapszulákból történő felszabadításának szabályozását is, érdemes vizsgálatokat elvégezni a mikrokapszulák erre vonatkozó karakterisztikájára és mechanizmusára is. Végezettül, érdemes lehet még tanulmányozni az *in vivo* vagy legalább egy dinamikus rendszerű *in vitro* emésztés hatását a

kapszulázott probiotikumok életképességére, valamint más polimerek vagy prebiotikum hatását is megvizsgálni a probiotikum hatékony hordozása szemszögéből.

REFERENCES (A1.)

AFZAAL, M., KHAN, A. U., SAEED, F., AHMED, A., AHMAD, M. H., MAAN, A. A., TUFAIL, T., ANJUM, F. M., & HUSSAIN, S. (2019): Functional exploration of free and encapsulated probiotic bacteria in yogurt and simulated gastrointestinal conditions. *Food Science and Nutrition*, *7*(12), 3931–3940. https://doi.org/10.1002/fsn3.1254

AHN, S. IL, CHANG, Y. H., & KWAK, H. S. (2010): Optimization of microencapsulation of inonotus obliquus extract powder by response surface methodology and its application into milk. *Korean Journal for Food Science of Animal Resources*, *30*(4), 661–668. https://doi.org/10.5851/kosfa.2010.30.4.661

AKHAVAN, S., & JAFARI, S. M. (2017): Nanoencapsulation of Natural Food Colorants. In: S. M. Jafari (Ed.), *Nanoencapsulation of Food Bioactive Ingredients* (pp. 223–260.).Elsevier. https://doi.org/10.1016/b978-0-12-809740-3.00006-4

ALLI, S. M. A., ALI, S. M. A., & SAMANTA, A. (2011): Development and evaluation of intestinal targeted mucoadhesive microspheres of Bacillus coagulans. *Drug Development and Industrial Pharmacy*, *37*(11), 1329–1338. https://doi.org/10.3109/03639045.2011.572889

AMARA, A. A., & SHIBL, A. (2015): Role of Probiotics in health improvement, infection control and disease treatment and management. *Saudi Pharmaceutical Journal*, *23*(2), 107–114. https://doi.org/10.1016/j.jsps.2013.07.001

ANAL, A. K., & SINGH, H. (2007): Recent advances in microencapsulation of probiotics for industrial applications and targeted delivery. *Trends in Food Science and Technology*, *18*(5), 240–251. https://doi.org/10.1016/j.tifs.2007.01.004

ANNAN, N. T., BORZA, A. D., & HANSEN, L. T. (2008): Encapsulation in alginate-coated gelatin microspheres improves survival of the probiotic *Bifidobacterium adolescentis* 15703T during exposure to simulated gastro-intestinal conditions. *Food Research International*, *41*(2), 184–193. https://doi.org/10.1016/j.foodres.2007.11.001

ANSELMO, A. C., MCHUGH, K. J., WEBSTER, J., LANGER, R., & JAKLENEC, A. (2016): Layer-by-Layer Encapsulation of Probiotics for Delivery to the Microbiome. *Advanced Materials*, *28*(43), 9486–9490. https://doi.org/10.1002/adma.201603270

ARGIN, S. (2007)*: Microencapsulation of Probiotic Bacteria in Xanthan-Chitosan Polyelectrolyte Complex Gels.* [University of Maryland]. https://drum.lib.umd.edu/handle/1903/7826

ARSLAN-TONTUL, S., & ERBAS, M. (2017): Single and double layered microencapsulation of probiotics by spray drying and spray chilling. *Lwt*, *81*, 160–169. https://doi.org/10.1016/j.lwt.2017.03.060

ASHAOLU, T. J. (2020): Immune boosting functional foods and their mechanisms: A critical evaluation of probiotics and prebiotics. *Biomedicine & Pharmacotherapy*, *130*, 110625. https://doi.org/10.1016/j.biopha.2020.110625

ASHWAR, B. A., GANI, A., GANI, A., SHAH, A., & MASOODI, F. A. (2018): Production of RS4 from rice starch and its utilization as an encapsulating agent for targeted delivery of probiotics. *Food Chemistry*, *239*, 287–294. https://doi.org/10.1016/j.foodchem.2017.06.110

ASPRI, M., PAPADEMAS, P., & TSALTAS, D. (2020): Review on non-dairy probiotics and their use in non-dairy based products. *Fermentation*, *6*(1), 1–20. https://doi.org/10.3390/fermentation6010030

ASTM STANDARD E799-03 (2015): Standard Practice for Determining Data Criteria and Processing

for Liquid Drop Size Analysis. *ASTM International, West Conshohocken, PA*, *03*(Reapproved 2015), 1–5. https://doi.org/10.1520/E0799-03R09.2

BAMPI, G. B., BACKES, G. T., CANSIAN, R. L., DE MATOS, F. E., ANSOLIN, I. M. A., POLETO, B. C., COREZZOLLA, L. R., & FAVARO-TRINDADE, C. S. (2016): Spray Chilling Microencapsulation of *Lactobacillus acidophilus* and *Bifidobacterium animalis subsp. lactis* and Its Use in the Preparation of Savory Probiotic Cereal Bars. *Food and Bioprocess Technology*, *9*(8), 1422–1428. https://doi.org/10.1007/s11947-016-1724-z

BANERJEE, S., & MAZUMDAR, S. (2012): Electrospray Ionization Mass Spectrometry: A Technique to Access the Information beyond the Molecular Weight of the Analyte. *International Journal of Analytical Chemistry*, *2012*, 1–40. https://doi.org/10.1155/2012/282574

BAYLESS, T. M., BROWN, E., & PAIGE, D. M. (2017): Lactase Non-persistence and Lactose Intolerance. *Current Gastroenterology Reports*, *19*(5), 23. https://doi.org/10.1007/s11894-017-0558-9

BEGLEY, M., HILL, C., & GAHAN, C. G. M. (2006): Bile Salt Hydrolase Activity in Probiotics. *Applied and Environmental Microbiology*, *72*(3), 1729–1738. https://doi.org/10.1128/AEM.72.3.1729-1738.2006

BHUSHANI, A., HARISH, U., & ANANDHARAMAKRISHNAN, C. (2017): Nanodelivery of nutrients for improved bioavailability. In: A. M. Grumezescu (Ed.), *Nutrient Delivery* (pp. 369–411.).Elsevier. https://doi.org/10.1016/B978-0-12-804304-2.00010-X

BLANCO, A., & BLANCO, G. (2017): Proteins. In: A. Blanco & G. Blanco (Eds.), *Medical Biochemistry* (pp. 21–71.).Elsevier. https://doi.org/10.1016/B978-0-12-803550-4.00003-3

BOONMA, P., SPINLER, J. K., QIN, X., JITTAPRASATSIN, C., MUZNY, D. M., DODDAPANENI, H., GIBBS, R., PETROSINO, J., TUMWASORN, S., & VERSALOVIC, J. (2015): Draft genome sequences and description of *Lactobacillus rhamnosus* strains L31, L34, and L35. *Standards in Genomic Sciences*, *9*(3), 744–754. https://doi.org/10.4056/sigs.5048907

BRACKER, R., & STENDER, A. S. (2019): Efficacy of USB microscopes for imaging forensic materials. *Forensic Science International*, *304*, 109961. https://doi.org/10.1016/j.forsciint.2019.109961

BRATCHER, D. F. (2018): Other Gram-Positive Bacilli. In: S. Long, C. Prober, & M. Fischer (Eds.), *Principles and Practice of Pediatric Infectious Diseases* (5th ed., pp. 786-790.e4.).Elsevier. https://doi.org/10.1016/B978-0-323-40181-4.00133-X

BRUCHET, M., & MELMAN, A. (2015): Fabrication of patterned calcium cross-linked alginate hydrogel films and coatings through reductive cation exchange. *Carbohydrate Polymers*, *131*, 57–64. https://doi.org/10.1016/j.carbpol.2015.05.021

BUTEL, M.-J. (2014): Probiotics, gut microbiota and health. *Médecine et Maladies Infectieuses*, *44*(1), 1–8. https://doi.org/10.1016/j.medmal.2013.10.002

BUTLER, N., & FLETCHER, J. (2019): Gluten-free foods for different diets. *Medical News Today*. https://www.medicalnewstoday.com/articles/324494.php

CĂLINOIU, L.-F., ŞTEFĂNESCU, B., POP, I., MUNTEAN, L., & VODNAR, D. (2019): Chitosan Coating Applications in Probiotic Microencapsulation. *Coatings*, *9*(3), 194. https://doi.org/10.3390/coatings9030194

CAMELIN, I., LACROIX, C., PAQUIN, C., PRÉVOST, H., CACHON, R., & DIVIES, C. (1993): Effect of Chelatants on Gellan Gel Rheological Properties and Setting Temperature for Immobilization of Living Bifidobacteria. *Biotechnology Progress*, *9*(3), 291–297. https://doi.org/10.1021/bp00021a008

CAPELEZZO, A. P., MOHR, L. C., DALCANTON, F., MELLO, J. M. M. DE, & FIORI, M. A. (2018): β-

Cyclodextrins as Encapsulating Agents of Essential Oils. In: P. Arora & N. Dhingra (Eds.), *Cyclodextrin - A Versatile Ingredient*.InTech. https://doi.org/10.5772/intechopen.73568

CHAIKHAM, P., & APICHARTSRANGKOON, A. (2012): Comparison of bioactive components in pressurized and pasteurized longan juices fortified with encapsulated *Lactobacillus casei* 01. *High Pressure Research*, *32*(2), 316–322. https://doi.org/10.1080/08957959.2012.665052

CHAKRABORTY, S. (2017): Carrageenan for encapsulation and immobilization of flavor, fragrance, probiotics, and enzymes: A review. *Journal of Carbohydrate Chemistry*, *36*(1), 1–19. https://doi.org/10.1080/07328303.2017.1347668

CHAMPAGNE, C. P., & GARDNER, N. J. (2008): Effect of storage in a fruit drink on subsequent survival of probiotic lactobacilli to gastro-intestinal stresses. *Food Research International*. https://doi.org/10.1016/j.foodres.2008.03.003

CHAN, E. S., & ZHANG, Z. (2005): Bioencapsulation by compression coating of probiotic bacteria for their protection in an acidic medium. *Process Biochemistry*, *40*(10), 3346–3351. https://doi.org/10.1016/j.procbio.2005.03.001

CHAN, ENG SENG (2011): Preparation of Ca-alginate beads containing high oil content: Influence of process variables on encapsulation efficiency and bead properties. *Carbohydrate Polymers*, *84*(4), 1267–1275. https://doi.org/10.1016/j.carbpol.2011.01.015

CHANDRAMOULI, V., KAILASAPATHY, K., PEIRIS, P., & JONES, M. (2004): An improved method of microencapsulation and its evaluation to protect *Lactobacillus* spp. in simulated gastric conditions. *Journal of Microbiological Methods*, *56*(1), 27–35. https://doi.org/10.1016/j.mimet.2003.09.002

CHANDRASEKARA, A. (2019): Phenolic Acids. In: L. Melton, F. Shahidi, & P. Varelis (Eds.), *Encyclopedia of Food Chemistry* (pp. 535–545.).Elsevier. https://doi.org/10.1016/B978-0-08-100596-5.22395-0

CHARTERIS, W. P., KELLY, P. M., MORELLI, L., & COLLINS, J. K. (1998): Development and application of an *in vitro* methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. *Journal of Applied Microbiology*, *84*(5), 759–768. https://doi.org/10.1046/j.1365-2672.1998.00407.x

CHÁVARRI, M., MARAÑÓN, I., ARES, R., IBÁÑEZ, F. C., MARZO, F., & VILLARÁN, M. DEL C. (2010): Microencapsulation of a probiotic and prebiotic in alginate-chitosan capsules improves survival in simulated gastro-intestinal conditions. *International Journal of Food Microbiology*, *142*(1–2), 185–189. https://doi.org/10.1016/j.ijfoodmicro.2010.06.022

CHAVARRI, M., MARANON, I., & CARMEN, M. (2012): Encapsulation Technology to Protect Probiotic Bacteria. *Probiotics*, 501–540. https://doi.org/10.5772/50046

CHEN, H.-Y., LI, X.-Y., LIU, B.-J., & MENG, X.-H. (2017): Microencapsulation of *Lactobacillus bulgaricus* and survival assays under simulated gastrointestinal conditions. *Journal of Functional Foods*, *29*, 248–255. https://doi.org/10.1016/j.jff.2016.12.015

CHEN, H., WANG, Y., SHU, G. W., & JIA, Y. L. (2012): Effect of Alginate and Cell Suspension on Viable Counts and Efficacy of Entrapment of Encapsulated *B. bifidum Bb28*. *Advanced Materials Research*, *531*, 499–502. https://doi.org/10.4028/www.scientific.net/AMR.531.499

CHEN, K. N., CHEN, M. J., LIU, J. E. R., LIN, C. W., & CHIU, H. Y. I. (2005): Optimization of incorporated prebiotics as coating materials for probiotic microencapsulation. *Journal of Food Science*, *70*(5), M260–M266. https://doi.org/10.1111/j.1365-2621.2005.tb09981.x

CHEN, M. J., & CHEN, K. N. (2007): Applications of Probiotic Encapsulation in Dairy Products. In: J.

M. Lakkis (Ed.), *Encapsulation and Controlled Release Technologies in Food Systems* (1st ed., pp. 83–112.).Wiley Blackwell. https://doi.org/10.1002/9780470277881.ch4

CHUNG, W. S. F., MEIJERINK, M., ZEUNER, B., HOLCK, J., LOUIS, P., MEYER, A. S., WELLS, J. M., FLINT, H. J., & DUNCAN, S. H. (2017): Prebiotic potential of pectin and pectic oligosaccharides to promote anti-inflammatory commensal bacteria in the human colon. *FEMS Microbiology Ecology*, *93*(11). https://doi.org/10.1093/femsec/fix127

CLEMENT, G. E., SIEGEL, A., & POTTER, R. (1971): Sodium Ion Binding to Pepsin and Other Proteins. *Canadian Journal of Biochemistry*, *49*(5), 477–483. https://doi.org/10.1139/o71-071

CLIFFORD, T., HOWATSON, G., WEST, D. J., & STEVENSON, E. J. (2015): The potential benefits of red beetroot supplementation in health and disease. *Nutrients*, *7*(4), 2801–2822. https://doi.org/10.3390/nu7042801

COGHETTO, C. C., BRINQUES, G. B., SIQUEIRA, N. M., PLETSCH, J., SOARES, R. M. D., & AYUB, M. A. Z. (2016): Electrospraying microencapsulation of *Lactobacillus plantarum* enhances cell viability under refrigeration storage and simulated gastric and intestinal fluids. *Journal of Functional Foods*, *24*, 316–326. https://doi.org/10.1016/j.jff.2016.03.036

CONLON, M. A., & BIRD, A. R. (2015): The impact of diet and lifestyle on gut microbiota and human health. *Nutrients*, *7*(1), 17–44. https://doi.org/10.3390/nu7010017

CONSIDINE, D. M., & CONSIDINE, G. D. (1995): Van Nostrand's Scientific Encyclopedia. In: G. D. Considine (Ed.), *Van Nostrand's Scientific Encyclopedia* (1st ed.).John Wiley & Sons, Inc. https://doi.org/10.1002/9780471743989

COOK, M. T., TZORTZIS, G., CHARALAMPOPOULOS, D., & KHUTORYANSKIY, V. V. (2011): Production and Evaluation of Dry Alginate-Chitosan Microcapsules as an Enteric Delivery Vehicle for Probiotic Bacteria. *Biomacromolecules*, *12*(7), 2834–2840. https://doi.org/10.1021/bm200576h

COOK, M. T., TZORTZIS, G., CHARALAMPOPOULOS, D., & KHUTORYANSKIY, V. V. (2012): Microencapsulation of probiotics for gastrointestinal delivery. *Journal of Controlled Release*, *162*(1), 56–67. https://doi.org/10.1016/j.jconrel.2012.06.003

COOK, S. L., BULL, S. P., METHVEN, L., PARKER, J. K., & KHUTORYANSKIY, V. V. (2017): Mucoadhesion: A food perspective. *Food Hydrocolloids*, *72*, 281–296. https://doi.org/10.1016/j.foodhyd.2017.05.043

COOK, S. L., WOODS, S., METHVEN, L., PARKER, J. K., & KHUTORYANSKIY, V. V. (2018): Mucoadhesive polysaccharides modulate sodium retention, release and taste perception. *Food Chemistry*, *240*, 482–489. https://doi.org/10.1016/j.foodchem.2017.07.134

CORCORAN, B. M., STANTON, C., FITZGERALD, G. F., & ROSS, R. P. (2005): Survival of probiotic lactobacilli in acidic environments is enhanced in the presence of metabolizable sugars. *Applied and Environmental Microbiology*, *71*(6), 3060–3067. https://doi.org/10.1128/AEM.71.6.3060-3067.2005

CORONA-HERNANDEZ, R. I., ÁLVAREZ-PARRILLA, E., LIZARDI-MENDOZA, J., ISLAS-RUBIO, A. R., DE LA ROSA, L. A., & WALL-MEDRANO, A. (2013): Structural stability and viability of microencapsulated probiotic bacteria: A review. *Comprehensive Reviews in Food Science and Food Safety*, *12*(6), 614–628. https://doi.org/10.1111/1541-4337.12030

CORSETTI, A., & GOBBETTI, M. (2002): *LACTOBACILLUS* spp. / *Lactobacillus plantarum*. In: H. Roginski (Ed.), *Encyclopedia of Dairy Sciences* (pp. 1501–1507.).Elsevier. https://doi.org/10.1016/b0-12-227235-8/00242-x

CORSETTI, A., & VALMORRI, S. (2011): Lactic Acid Bacteria: *Lactobacillus* spp.: *Lactobacillus*

plantarum. In: J. W. Fuquay (Ed.), *Encyclopedia of Dairy Sciences: Second Edition* (pp. 111–118.).Academic Press. https://doi.org/10.1016/B978-0-12-374407-4.00263-6

COSTA, P., ROCHA-SANTOS, T., GOMES, A., PINTADO, M., SOUSA, S., AMARAL, M., & SOUSA E SILVA, J. (2014): Immobilization and Microencapsulation of Probiotics. In: J. P. Sousa e Silva & A. C. Freitas (Eds.), *Probiotic Bacteria* (pp. 171–226.).Pan Stanford Publishing. https://doi.org/10.1201/b15676-6

COTTER, P. D., & HILL, C. (2003): Surviving the Acid Test: Responses of Gram-Positive Bacteria to Low pH. *Microbiology and Molecular Biology Reviews*, *67*(3), 429–453. https://doi.org/10.1128/MMBR.67.3.429-453.2003

CUMMINS, J., & HO, M. W. (2005): Genetically modified probiotics should be banned. *Microbial Ecology in Health and Disease*, *17*(2), 66–68. https://doi.org/10.1080/08910600510044480

DAVANI-DAVARI, D., NEGAHDARIPOUR, M., KARIMZADEH, I., SEIFAN, M., MOHKAM, M., MASOUMI, S., BERENJIAN, A., & GHASEMI, Y. (2019): Prebiotics: Definition, Types, Sources, Mechanisms, and Clinical Applications. *Foods*, *8*(3), 92. https://doi.org/10.3390/foods8030092

DE MAN, J. C., ROGOSA, M., & SHARPE, M. E. (1960): a Medium for the Cultivation of Lactobacilli. *Journal of Applied Bacteriology*, *23*(1), 130–135. https://doi.org/10.1111/j.1365-2672.1960.tb00188.x

DE PRISCO, A., & MAURIELLO, G. (2016): Probiotication of foods: A focus on microencapsulation tool. *Trends in Food Science and Technology*, *48*, 27–39. https://doi.org/10.1016/j.tifs.2015.11.009

DESMOND, C., ROSS, R. P., O'CALLAGHAN, E., FITZGERALD, G., & STANTON, C. (2002): Improved survival of *Lactobacillus paracasei* NFBC 338 in spray-dried powders containing gum acacia. *Journal of Applied Microbiology*, *93*(6), 1003–1011. https://doi.org/10.1046/j.1365-2672.2002.01782.x

DEY, G. (2018): Non-dairy Probiotic Foods: Innovations and Market Trends. In: S. K. Panda & P. H. Shetty (Eds.), *Innovations in Technologies for Fermented Food and Beverage Industries* (pp. 159–173.).Springer International Publishing. https://doi.org/10.1007/978-3-319-74820-7_9

DIANAWATI, D., MISHRA, V., & SHAH, N. P. (2016): Survival of Microencapsulated Probiotic Bacteria after Processing and during Storage: A Review. *Critical Reviews in Food Science and Nutrition*, *56*(10), 1685–1716. https://doi.org/10.1080/10408398.2013.798779

DIASPRO, A., SILVANO, D., KROL, S., CAVALLERI, O., & GLIOZZI, A. (2002): Single living cell encapsulation in nano-organized polyelectrolyte shells. *Langmuir*, *18*(13), 5047–5050. https://doi.org/10.1021/la025646e

DÍEZ-MUNICIO, M., HERRERO, M., OLANO, A., & MORENO, F. J. (2014): Synthesis of novel bioactive lactose-derived oligosaccharides by microbial glycoside hydrolases. *Microbial Biotechnology*, *7*(4), 315–331. https://doi.org/10.1111/1751-7915.12124

DIKIT, P., H-KITTIKUN, A., & MANEERAT, S. (2015): Survival of encapsulated potentially probiotic *Lactobacillus plantarum* D6SM3 with bioemulsifier derived from spent yeast in simulated gastrointestinal conditions. *Songklanakarin Journal of Science and Technology*, *37*(4), 425–432.

DINAKAR, P., & MISTRY, V. V. (1994): Growth and Viability of *Bifidobacterium bifidum* in Cheddar Cheese. *Journal of Dairy Science*, *77*(10), 2854–2864. https://doi.org/10.3168/jds.S0022-0302(94)77225-8

DING, W. K., & SHAH, N. P. (2007): Acid, bile, and heat tolerance of free and microencapsulated probiotic bacteria. *Journal of Food Science*, *72*(9), M446–M450. https://doi.org/10.1111/j.1750-

3841.2007.00565.x

DO CARMO, M. S., SANTOS, C. ITAPARY DOS, ARAÚJO, M. C., GIRÓN, J. A., FERNANDES, E. S., & MONTEIRO-NETO, V. (2018): Probiotics, mechanisms of action, and clinical perspectives for diarrhea management in children. *Food & Function*, *9*(10), 5074–5095. https://doi.org/10.1039/C8FO00376A

DODOO, C. C., WANG, J., BASIT, A. W., STAPLETON, P., & GAISFORD, S. (2017): Targeted delivery of probiotics to enhance gastrointestinal stability and intestinal colonisation. *International Journal of Pharmaceutics*, *530*(1–2), 224–229. https://doi.org/10.1016/j.ijpharm.2017.07.068

DONOHUE, D. C., & GUEIMONDE, M. (2011): Some Considerations for the Safety of Novel Probiotic Bacteria. In: L. Sampo, O. Arthur C., S. Seppo, & von W. Atte (Eds.), *Lactic Acid Bacteria* (4th ed., pp. 441–456.).CRC Press. https://doi.org/10.1201/b11503-25

DRAGET, K. I., SKJÅK BRÆK, G., & SMIDSRØD, O. (1994): Alginic acid gels: the effect of alginate chemical composition and molecular weight. *Carbohydrate Polymers*, *25*(1), 31–38. https://doi.org/10.1016/0144-8617(94)90159-7

EGGER, L., MÉNARD, O., DELGADO-ANDRADE, C., ALVITO, P., ASSUNÇÃO, R., BALANCE, S., BARBERÁ, R., BRODKORB, A., CATTENOZ, T., CLEMENTE, A., COMI, I., DUPONT, D., GARCIA-LLATAS, G., LAGARDA, M. J., LE FEUNTEUN, S., JANSSENDUIJGHUIJSEN, L., KARAKAYA, S., LESMES, U., MACKIE, A. R., … PORTMANN, R. (2016): The harmonized INFOGEST *in vitro* digestion method: From knowledge to action. *Food Research International*, *88*, 217–225. https://doi.org/10.1016/j.foodres.2015.12.006

EL-ABD, M. M., ABDELHAMID, M., EL-SAYED, H., A.EL-METWALY, H., EL-DEMERDASH, M. E., & MOHAMED, Z. (2018): Viability of Microencapsulated Probiotics Combined with Plant Extracts in Fermented Camel Milk under Simulated Gastrointestinal Conditions. *Middle East Journal of Applied Sciences*, *08*(03), 837–850.

ELZATAHRY, A. A., ELDIN, M. S. M., SOLIMAN, E. A., & HASSAN, E. A. (2009): Evaluation of alginate-chitosan bioadhesive beads as a drug delivery system for the controlled release of theophylline. *Journal of Applied Polymer Science*, *111*(5), 2452–2459. https://doi.org/10.1002/app.29221

ERATTE, D., DOWLING, K., BARROW, C. J., & ADHIKARI, B. P. (2017): In-vitro digestion of probiotic bacteria and omega-3 oil co-microencapsulated in whey protein isolate-gum Arabic complex coacervates. *Food Chemistry*, *227*, 129–136. https://doi.org/10.1016/j.foodchem.2017.01.080

ESKE, J., & MARENGO, K. (2019)*Beetroot juice: 6 health benefits, nutrition, and how to use it*Medical News Today. https://www.medicalnewstoday.com/articles/324898#_noHeaderPrefixedContent

ESKESEN, D., JESPERSEN, L., MICHELSEN, B., WHORWELL, P. J., MÜLLER-LISSNER, S., & MORBERG, C. M. (2015): Effect of the probiotic strain *Bifidobacterium animalis subsp. lactis*, Bb-12®, on defecation frequency in healthy subjects with low defecation frequency and abdominal discomfort: A randomised, double-blind, placebo-controlled, parallel-group trial. *British Journal of Nutrition*, *114*(10), 1638–1646. https://doi.org/10.1017/S0007114515003347

FARDET, A. (2017): New Concepts and Paradigms for the Protective Effects of Plant-Based Food Components in Relation to Food Complexity. In: F. Mariotti (Ed.), *Vegetarian and Plant-Based Diets in Health and Disease Prevention* (pp. 293–312.).Elsevier. https://doi.org/10.1016/B978-0-12-803968-7.00016-2

FASOLIN, L. H., PICONE, C. S. F., SANTANA, R. C., & CUNHA, R. L. (2013): Production of hybrid gels from polysorbate and gellan gum. *Food Research International*, *54*(1), 501–507. https://doi.org/10.1016/j.foodres.2013.07.026

FERRARIO, C., TURRONI, F., SINDEREN, D. VAN, & VENTURA, M. (2019): Bifidobacteria. In: G. Vinderola, A. C. Ouwehand, S. Salminen, & A. von Wright (Eds.), *Lactic Acid Bacteria* (5th ed., pp. 125–137.).CRC Press. https://doi.org/10.1201/9780429057465-9

FRAKOLAKI, G., GIANNOU, V., KEKOS, D., & TZIA, C. (2020): A review of the microencapsulation techniques for the incorporation of probiotic bacteria in functional foods. *Critical Reviews in Food Science and Nutrition*, 1–22. https://doi.org/10.1080/10408398.2020.1761773

FREDUA-AGYEMAN, M., & GAISFORD, S. (2015): Comparative survival of commercial probiotic formulations: Tests in biorelevant gastric fluids and real-time measurements using microcalorimetry. *Beneficial Microbes*, *6*(1), 141–151. https://doi.org/10.3920/BM2014.0051

FREDUA-AGYEMAN, MANSA, STAPLETON, P., BASIT, A. W., BEEZER, A. E., & GAISFORD, S. (2017): *In vitro* inhibition of Clostridium difficile by commercial probiotics: A microcalorimetric study. *International Journal of Pharmaceutics*, *517*(1–2), 96–103. https://doi.org/10.1016/j.ijpharm.2016.12.005

FULLER, R., & GIBSON, G. R. (1997): Modification of the Intestinal Microflora Using Probiotics and Prebiotics. *Scandinavian Journal of Gastroenterology*, *32*(sup222), 28–31. https://doi.org/10.1080/00365521.1997.11720714

GÄNZLE, M. G. (2011): Lactose and Oligosaccharides: Lactose: Derivatives. In: J. W. Fuquay (Ed.), *Encyclopedia of Dairy Sciences: Second Edition* (2nd ed., pp. 202–208.).Elsevier. https://doi.org/10.1016/B978-0-12-374407-4.00275-2

GARDINER, G. E., BOUCHIER, P., O'SULLIVAN, E., KELLY, J., KEVIN COLLINS, J., FITZGERALD, G., PAUL ROSS, R., & STANTON, C. (2002): A spray-dried culture for probiotic Cheddar cheese manufacture. *International Dairy Journal*, *12*(9), 749–756. https://doi.org/10.1016/S0958-6946(02)00072-9

GASBARRINI, G., BONVICINI, F., & GRAMENZI, A. (2016): Probiotics History. *Journal of Clinical Gastroenterology*, *50*, S116–S119. https://doi.org/10.1097/MCG.0000000000000697

GAWKOWSKI, D., & CHIKINDAS, M. L. (2013): Non-dairy probiotic beverages: The next step into human health. *Beneficial Microbes*, *4*(2), 127–142. https://doi.org/10.3920/BM2012.0030

GBASSI, G. K., VANDAMME, T., ENNAHAR, S., & MARCHIONI, E. (2009): Microencapsulation of *Lactobacillus plantarum* spp in an alginate matrix coated with whey proteins. *International Journal of Food Microbiology*, *129*(1), 103–105. https://doi.org/10.1016/j.ijfoodmicro.2008.11.012

GBASSI, GILDAS K., & VANDAMME, T. (2012): Probiotic encapsulation technology: From microencapsulation to release into the gut. *Pharmaceutics*, *4*(1), 149–163. https://doi.org/10.3390/pharmaceutics4010149

GIRALDO, J. D., CAMPOS-REQUENA, V. H., & RIVAS, B. L. (2019): Chitosan–tripolyphosphate bead: the interactions that govern its formation. *Polymer Bulletin*, *76*(8), 3879–3903. https://doi.org/10.1007/s00289-018-2574-9

GOBBETTI, M., & MINERVINI, F. (2014): *Lactobacillus*: *Lactobacillus casei*. In: C. A. Batt & M. Lou Tortorello (Eds.), *Encyclopedia of Food Microbiology: Second Edition* (pp. 432–438.).Academic Press. https://doi.org/10.1016/B978-0-12-384730-0.00180-4

GOKAVI, S., ZHANG, L., HUANG, M.-K., ZHAO, X., & GUO, M. (2006): Oat-based Symbiotic Beverage Fermented by *Lactobacillus plantarum*, *Lactobacillus paracasei* ssp. *casei*, and *Lactobacillus acidophilus*. *Journal of Food Science*, *70*(4), M216–M223. https://doi.org/10.1111/j.1365-2621.2005.tb07191.x

GOMBOTZ, W. R., & WEE, S. F. (2012): Protein release from alginate matrices. *Advanced Drug Delivery Reviews*, *64*(SUPPL.), 194–205. https://doi.org/10.1016/j.addr.2012.09.007

GOMES, A., PINTADO, M., & SOUSA E SILVA, J. (2014): Gastrointestinal Tract: Gastrointestinal Tract: Microflora and Transit Aspects. In: J. P. S. e Silva & A. C. Freitas (Eds.), *Probiotic Bacteria* (pp. 7–45.).Pan Stanford Publishing. https://doi.org/10.1201/b15676-3

GÓMEZ-MASCARAQUE, L. G., AMBROSIO-MARTÍN, J., PEREZ-MASIÁ, R., & LOPEZ-RUBIO, A. (2017): Impact of Acetic Acid on the Survival of *L. plantarum* upon Microencapsulation by Coaxial Electrospraying. *Journal of Healthcare Engineering*, *2017*, 1–6. https://doi.org/10.1155/2017/4698079

GOMEZ-MASCARAQUE, L. G., MORFIN, R. C., PÉREZ-MASIÁ, R., SANCHEZ, G., & LOPEZ-RUBIO, A. (2016): Optimization of electrospraying conditions for the microencapsulation of probiotics and evaluation of their resistance during storage and in-vitro digestion. *LWT - Food Science and Technology*, *69*, 438–446. https://doi.org/10.1016/j.lwt.2016.01.071

GUL, O., & DERVISOGLU, M. (2017): Application of multicriteria decision technique to determine optimum sodium alginate concentration for microencapsulation of *Lactobacillus casei* Shirota by extrusion and emulsification. *Journal of Food Process Engineering*, *40*(3), e12481. https://doi.org/10.1111/jfpe.12481

GUZMÁN, E., MATEOS-MAROTO, A., RUANO, M., ORTEGA, F., & RUBIO, R. G. (2017): Layer-by-Layer polyelectrolyte assemblies for encapsulation and release of active compounds. *Advances in Colloid and Interface Science*, *April*, 0–1. https://doi.org/10.1016/j.cis.2017.04.009

HADZIEVA, J., MLADENOVSKA, K., SIMONOSKA CRCAREVSKA, M., GLAVAŠ DODOV, M., DIMCHEVSKA, S., GEŠKOVSKI, N., GROZDANOV, A., POPOVSKI, E., PETRUŠEVSKI, G., CHACHOROVSKA, M., PETRESKA IVANOVSKA, T., PETRUŠEVSKA-TOZI, L., UGARKOVIC, S., & GORACINOVA, K. (2017): *Lactobacillus casei* loaded Soy Protein-Alginate Microparticles prepared by Spray-Drying. *Food Technology and Biotechnology*, *55*(2). https://doi.org/10.17113/ftb.55.02.17.4991

HAFFNER, F. B., DIAB, R., & PASC, A. (2016): Encapsulation of probiotics: Insights into academic and industrial approaches. *AIMS Materials Science*, *3*(1), 114–136. https://doi.org/10.3934/matersci.2016.1.114

HAFFNER, F. B., VAN DE WIELE, T., & PASC, A. (2017): Original behavior of: *L. rhamnosus* GG encapsulated in freeze-dried alginate-silica microparticles revealed under simulated gastrointestinal conditions. *Journal of Materials Chemistry B*, *5*(38), 7839–7847. https://doi.org/10.1039/c7tb02190a

HAGHSHENAS, B., ABDULLAH, N., NAMI, Y., RADIAH, D., ROSLI, R., & YARI KHOSROUSHAHI, A. (2015): Microencapsulation of probiotic bacteria *Lactobacillus plantarum* 15HN using alginate-psyllium-fenugreek polymeric blends. *Journal of Applied Microbiology*, *118*(4), 1048–1057. https://doi.org/10.1111/jam.12762

HAY, A. J., & ZHU, J. (2016): In Sickness and in Health: The Relationships Between Bacteria and Bile in the Human Gut. *Advances in Applied Microbiology*, *96*, 43–64. https://doi.org/10.1016/bs.aambs.2016.07.019

HENNING, S. M., YANG, J., SHAO, P., LEE, R.-P., HUANG, J., LY, A., HSU, M., LU, Q.-Y., THAMES, G., HEBER, D., & LI, Z. (2017): Health benefit of vegetable/fruit juice-based diet: Role of microbiome. *Scientific Reports*, *7*(1), 2167. https://doi.org/10.1038/s41598-017-02200-6

HENRIQUE RODRIGUES DO AMARAL, P., LOPES ANDRADE, P., & COSTA DE CONTO, L. (2019): Microencapsulation and Its Uses in Food Science and Technology: A Review. In: F. Salaün (Ed.), *Microencapsulation - Processes, Technologies and Industrial Applications*.IntechOpen.

https://doi.org/10.5772/intechopen.81997

HERBEL, S. R., VAHJEN, W., WIELER, L. H., & GUENTHER, S. (2013): Timely approaches to identify probiotic species of the genus Lactobacillus. *Gut Pathogens*, *5*(1), 27. https://doi.org/10.1186/1757-4749-5-27

HILL, D., SUGRUE, I., TOBIN, C., HILL, C., STANTON, C., & ROSS, R. P. (2018): The *Lactobacillus casei* Group: History and Health Related Applications. *Frontiers in Microbiology*, *9*(SEP), 2107. https://doi.org/10.3389/fmicb.2018.02107

HOLKEM, A. T., RADDATZ, G. C., BARIN, J. S., MORAES FLORES, É. M., MULLER, E. I., CODEVILLA, C. F., JACOB-LOPES, E., FERREIRA GROSSO, C. R., & DE MENEZES, C. R. (2017): Production of microcapsules containing *Bifidobacterium* BB-12 by emulsification/internal gelation. *LWT - Food Science and Technology*, *76*, 216–221. https://doi.org/10.1016/j.lwt.2016.07.013

HOLKEM, A. T., RADDATZ, G. C., NUNES, G. L., CICHOSKI, A. J., JACOB-LOPES, E., FERREIRA GROSSO, C. R., & DE MENEZES, C. R. (2016): Development and characterization of alginate microcapsules containing *Bifidobacterium* BB-12 produced by emulsification/internal gelation followed by freeze drying. *LWT - Food Science and Technology*, *71*, 302–308. https://doi.org/10.1016/j.lwt.2016.04.012

HOLTZAPPLE, M. T. (2003): CELLULOSE. In: B. Caballero (Ed.), *Encyclopedia of Food Sciences and Nutrition* (2nd ed., pp. 998–1007.).Elsevier. https://doi.org/10.1016/B0-12-227055-X/00185-1

HOOVER, D.G. (2014): *Bifidobacterium*. In: C. A. Batt & M. Lou Tortorello (Eds.), *Encyclopedia of Food Microbiology* (2nd ed., pp. 216–222.).Elsevier. https://doi.org/10.1016/B978-0-12-384730-0.00033-1

IBRAHIM, F., & OUWEHAND, A. C. (2019): The Genus Lactobacillus. In: G. Vinderola, A. C. Ouwehand, S. Salminen, & A. von Wright (Eds.), *Lactic Acid Bacteria* (pp. 47–56.).CRC Press. https://doi.org/10.1201/9780429057465-4

IYER, C., & KAILASAPATHY, K. (2005): Effect of co-encapsulation of probiotics with prebiotics on increasing the viability of encapsulated bacteria under *in vitro* acidic and bile salt conditions and in yogurt. *Journal of Food Science*, *70*(1), M18–M23. https://doi.org/10.1111/j.1365-2621.2005.tb09041.x

JAFARI-NODOUSHAN, M., MOBEDI, H., & BARZIN, J. (2015): Encapsulation via Electrohydrodynamic Atomization Spray Technology (Electrospray). In: M. Mishra (Ed.), *Handbook of Encapsulation and Controlled Release* (1st ed., pp. 411–433.).CRC Press. https://doi.org/10.1201/b19038-29

JELKMANN, M., LEICHNER, C., MENZEL, C., KREB, V., & BERNKOP-SCHNÜRCH, A. (2019): Cationic starch derivatives as mucoadhesive and soluble excipients in drug delivery. *International Journal of Pharmaceutics*, *570*(July), 118664. https://doi.org/10.1016/j.ijpharm.2019.118664

JI, R., WU, J., ZHANG, J., WANG, T., ZHANG, X., SHAO, L., CHEN, D., & WANG, J. (2019): Extending viability of *Bifidobacterium longum* in chitosan-coated alginate microcapsules using emulsification and internal gelation encapsulation technology. *Frontiers in Microbiology*, *10*(JUN). https://doi.org/10.3389/fmicb.2019.01389

JIMÉNEZ-PRANTEDA, M. L., PONCELET, D., NÁDER-MACÍAS, M. E., ARCOS, A., AGUILERA, M., MONTEOLIVA-SÁNCHEZ, M., & RAMOS-CORMENZANA, A. (2012): Stability of lactobacilli encapsulated in various microbial polymers. *Journal of Bioscience and Bioengineering*. https://doi.org/10.1016/j.jbiosc.2011.10.010

JINDAL, N., & SINGH KHATTAR, J. (2018): Microbial Polysaccharides in Food Industry. In: A. M. Grumezescu & A. M. Holban (Eds.), *Biopolymers for Food Design* (pp. 95–123.).Elsevier.

https://doi.org/10.1016/B978-0-12-811449-0.00004-9

JUNGERSEN, M., WIND, A., JOHANSEN, E., CHRISTENSEN, J., STUER-LAURIDSEN, B., & ESKESEN, D. (2014): The Science behind the Probiotic Strain *Bifidobacterium animalis subsp. lactis* Bb-12®. *Microorganisms*, *2*(2), 92–110. https://doi.org/10.3390/microorganisms2020092

KAILASAPATHY, K. (2006): Survival of free and encapsulated probiotic bacteria and their effect on the sensory properties of yoghurt. *LWT - Food Science and Technology*, *39*(10), 1221–1227. https://doi.org/10.1016/j.lwt.2005.07.013

KAILASAPATHY, KAILA (2002): Microencapsulation of Probiotic Bacteria 39 Microencapsulation of Probiotic Bacteria: Technology and Potential Applications. *Issues Intest. Microbiol*, *3*, 39–48. www.caister.com/bacteria-plant

KALDYBEKOV, D. B., TONGLAIROUM, P., OPANASOPIT, P., & KHUTORYANSKIY, V. V. (2018): Mucoadhesive maleimide-functionalised liposomes for drug delivery to urinary bladder. *European Journal of Pharmaceutical Sciences*, *111*, 83–90. https://doi.org/10.1016/j.ejps.2017.09.039

KANDYLIS, P., PISSARIDI, K., BEKATOROU, A., KANELLAKI, M., & KOUTINAS, A. A. (2016): Dairy and non-dairy probiotic beverages. *Current Opinion in Food Science*, *7*, 58–63. https://doi.org/10.1016/j.cofs.2015.11.012

KAUSAR, H., SAEED, S., AHMAD, M., & SALAM, A. (2012): Studies on the development and storage stability of cucumber-melon functional drink. *J. Agric. Res*, *50*, 239–248. https://doi.org/10.13140/RG.2.2.35674.18886

KHANDBAHALE, S. V. (2020): Microencapsulation-A Novel Approach in Drug Delivery: A Review. *Asian Journal of Research in Pharmaceutical Science*, *10*(1), 39. https://doi.org/10.5958/2231-5659.2020.00009.0

KHODER, G., AL-MENHALI, A. A., AL-YASSIR, F., & KARAM, S. M. (2016): Potential role of probiotics in the management of gastric ulcer (Review). *Experimental and Therapeutic Medicine*, *12*(1), 3–17. https://doi.org/10.3892/etm.2016.3293

KHOSRAVI ZANJANI, M. A., TARZI, B. G., SHARIFAN, A., & MOHAMMADI, N. (2014): Microencapsulation of probiotics by calcium alginate-gelatinized starch with chitosan coating and evaluation of survival in simulated human gastro-intestinal condition. *Iranian Journal of Pharmaceutical Research*, *13*(3), 843–852. https://doi.org/10.22037/ijpr.2014.1550

KHUTORYANSKIY, V. V. (2011): Advances in mucoadhesion and mucoadhesive polymers. *Macromolecular Bioscience*, *11*(6), 748–764. https://doi.org/10.1002/mabi.201000388

KHUTORYANSKIY, V. V. (2014): Mucoadhesive Materials and Drug Delivery Systems. In: V. V. Khutoryanskiy (Ed.), *Mucoadhesive Materials and Drug Delivery Systems* (Vol. 9781119941).John Wiley & Sons, Ltd. https://doi.org/10.1002/9781118794203

KILDEEVA, N. R., PERMINOV, P. A., VLADIMIROV, L. V., NOVIKOV, V. V., & MIKHAILOV, S. N. (2009): About mechanism of chitosan cross-linking with glutaraldehyde. *Russian Journal of Bioorganic Chemistry*, *35*(3), 360–369. https://doi.org/10.1134/S106816200903011X

KOGA, Y., OHTSU, T., KIMURA, K., & ASAMI, Y. (2019): Probiotic *L. gasseri* strain (LG21) for the upper gastrointestinal tract acting through improvement of indigenous microbiota. *BMJ Open Gastroenterology*, *6*(1), e000314. https://doi.org/10.1136/bmjgast-2019-000314

KOMATI, S., SWAIN, S., RAO, M. E. B., JENA, B. R., & DASI, V. (2019): Mucoadhesive Multiparticulate Drug Delivery Systems: An ExtensiveReview of Patents. *Advanced Pharmaceutical Bulletin*, *9*(4), 521–538. https://doi.org/10.15171/apb.2019.062

KRASAEKOOPT, W., & WATCHARAPOKA, S. (2014): Effect of addition of inulin and galactooligosaccharide on the survival of microencapsulated probiotics in alginate beads coated with chitosan in simulated digestive system, yogurt and fruit juice. *LWT - Food Science and Technology*, *57*(2), 761–766. https://doi.org/10.1016/j.lwt.2014.01.037

KRASAEKOOPT, WUNWISA (2013): Microencapsulation of probiotics in hydrocolloid gel matrices: A review. *Agro Food Industry Hi-Tech*, *24*(2), 76–83.

KRASAEKOOPT, WUNWISA, BHANDARI, B., & DEETH, H. (2003): Evaluation of encapsulation techniques of probiotics for yoghurt. *International Dairy Journal*, *13*(1), 3–13. https://doi.org/10.1016/S0958-6946(02)00155-3

KRASAEKOOPT, WUNWISA, BHANDARI, B., & DEETH, H. (2004): The influence of coating materials on some properties of alginate beads and survivability of microencapsulated probiotic bacteria. *International Dairy Journal*, *14*(8), 737–743. https://doi.org/10.1016/j.idairyj.2004.01.004

KULKARNI, V. S., & SHAW, C. (2016): Use of Polymers and Thickeners in Semisolid and Liquid Formulations. In: V. S. Kulkarni & C. Shaw (Eds.), *Essential Chemistry for Formulators of Semisolid and Liquid Dosages* (pp. 43–69.).Elsevier. https://doi.org/10.1016/B978-0-12-801024-2.00005-4

KUMAR, B. V., VIJAYENDRA, S. V. N., & REDDY, O. V. S. (2015): Trends :dairy and non-dairy probiotic products - a review. *Journal of Food Science and Technology*, *52*(10), 6112–6124. https://doi.org/10.1007/s13197-015-1795-2

KUMAR, H., & SALMINEN, S. (2016): Probiotics. In: B. Caballero, P. M. Finglas, & F. Toldrá (Eds.), *Encyclopedia of Food and Health* (pp. 510–515.).Elsevier Inc. https://doi.org/10.1016/B978-0-12-384947-2.00570-5

KURDI, P., KAWANISHI, K., MIZUTANI, K., & YOKOTA, A. (2006): Mechanism of growth inhibition by free bile acids in lactobacilli and bifidobacteria. *Journal of Bacteriology*, *188*(5), 1979–1986. https://doi.org/10.1128/JB.188.5.1979-1986.2006

KWAK, H. S. (2014): Overview of Nano- and Microencapsulation for Foods. In: H.-S. Kwak (Ed.), *Nano- and Microencapsulation for Foods* (Vol. 9781118292, pp. 1–14.).John Wiley & Sons, Ltd. https://doi.org/10.1002/9781118292327.ch1

LAELORSPOEN, N., WONGSASULAK, S., YOOVIDHYA, T., & DEVAHASTIN, S. (2014): Microencapsulation of *Lactobacillus acidophilus* in zein-alginate core-shell microcapsules via electrospraying. *Journal of Functional Foods*, *7*(1), 342–349. https://doi.org/10.1016/j.jff.2014.01.026

LANDETE, J. M., RODRIGUEZ, H., CURIEL, J. A., DE LAS RIVAS, B., DE FELIPE, F. L., & MUÑOZ, R. (2010): Degradation of Phenolic Compounds Found in Olive Products by *Lactobacillus plantarum* Strains. In: V. R. Preedy & R. R. Watson (Eds.), *Olives and Olive Oil in Health and Disease Prevention* (pp. 387–396.).Academic Press. https://doi.org/10.1016/B978-0-12-374420-3.00043-7

LANKAPUTHRA, W. E. V., & SHAH, N. P. (1995): Survival of *Lactobacillus acidophilus* and *Bifidobacterium* spp in the presence of acid and bile salts. *Cultured Dairy Products Journal*, *30*(3), 2–7. http://europepmc.org/abstract/AGR/IND20486713

LEE, H., SONG, C., BAIK, S., KIM, D., HYEON, T., & KIM, D.-H. (2018): Device-assisted transdermal drug delivery. *Advanced Drug Delivery Reviews*, *127*, 35–45. https://doi.org/10.1016/j.addr.2017.08.009

LEE, J. S., CHA, D. S., & PARK, H. J. (2004): Survival of freeze-dried *Lactobacillus bulgaricus* KFRI

673 in chitosan-coated calcium alginate microparticles. *Journal of Agricultural and Food Chemistry*, *52*(24), 7300–7305. https://doi.org/10.1021/jf040235k

LEE, K. Y., & HEO, T. R. (2000): Survival of *Bifidobacterium longum* immobilized in calcium alginate beads in simulated gastric juices and bile salt solution. *Applied and Environmental Microbiology*, *66*(2), 869–873. https://doi.org/10.1128/AEM.66.2.869-873.2000

LEVY, E. S., & SHAH, K. H. (2011): Liver and biliary tract disease. In: V. J. Markovchick, P. T. Pons, & K. M. Bakes (Eds.), *Emergency Medicine Secrets* (5th ed., pp. 248–254.).Elsevier. https://doi.org/10.1016/B978-0-323-07167-3.00044-9

LI, H., LI, C., ZHANG, T., HAN, Y., LI, H., & YU, J. (2020): Preparation of *Lactobacillus casei*/Lactitol Synbiotic Microcapsule and Its Application in Orange Juice. *Journal of Chinese Institute of Food Science and Technology*, *20*(4), 93–99. https://doi.org/10.16429/j.1009-7848.2020.04.013

LI, S. C., HSU, W. F., CHANG, J. S., & SHIH, C. K. (2019): Combination of *Lactobacillus acidophilus* and *Bifidobacterium animalis subsp. lactis* shows a stronger anti-inflammatory effect than individual strains in HT-29 cells. *Nutrients*. https://doi.org/10.3390/nu11050969

LI, X. Y., CHEN, X. G., SUN, Z. W., PARK, H. J., & CHA, D. S. (2011): Preparation of alginate/chitosan/carboxymethyl chitosan complex microcapsules and application in *Lactobacillus casei* ATCC 393. *Carbohydrate Polymers*, *83*(4), 1479–1485. https://doi.org/10.1016/j.carbpol.2010.09.053

LIAO, N., LUO, B., GAO, J., LI, X., ZHAO, Z., ZHANG, Y., NI, Y., & TIAN, F. (2019): Oligosaccharides as co-encapsulating agents: effect on oral *Lactobacillus fermentum* survival in a simulated gastrointestinal tract. *Biotechnology Letters*. https://doi.org/10.1007/s10529-018-02634-6

LIN, T. C., CHEN, B. Y., CHEN, C. Y., CHEN, Y. S., & WU, H. (2019): Comparative analysis of spray-drying microencapsulation of *Bifidobacterium adolescentis* and *Lactobacillus acidophilus* cultivated in different growth media. *Journal of Food Process Engineering*, *42*(7). https://doi.org/10.1111/jfpe.13258

LISERRE, A. M., RÉ, M. I., & FRANCO, B. D. G. M. (2007): Microencapsulation of *Bifidobacterium animalis subsp. lactis* in Modified Alginate-chitosan Beads and Evaluation of Survival in Simulated Gastrointestinal Conditions. *Food Biotechnology*, *21*(1), 1–16. https://doi.org/10.1080/08905430701191064

LIU, H., XIE, M., & NIE, S. (2020): Recent trends and applications of polysaccharides for microencapsulation of probiotics. *Food Frontiers*, *1*(1), 45–59. https://doi.org/10.1002/fft2.11

LOBO, V., PATIL, A., PHATAK, A., & CHANDRA, N. (2010): Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacognosy Reviews*, *4*(8), 118. https://doi.org/10.4103/0973-7847.70902

LOCKWOOD, A. H. (2008): Hepatic Encephalopathy. In: M. J. Aminoff (Ed.), *Neurology and General Medicine* (4th ed., pp. 265–279.).Elsevier. https://doi.org/10.1016/B978-044306707-5.50017-1

LONG, J., PAN, T., XIE, Z., XU, X., & JIN, Z. (2019): Effective production of lactosucrose using β-fructofuranosidase and glucose oxidase co-immobilized by sol–gel encapsulation. *Food Science and Nutrition*, *7*(10), 3302–3316. https://doi.org/10.1002/fsn3.1195

LORENZO, J. M., MUNEKATA, P. E., DOMINGUEZ, R., PATEIRO, M., SARAIVA, J. A., & FRANCO, D. (2018): Main groups of microorganisms of relevance for food safety and stability: General aspects and overall description. In: F. J. Barba, A. S. Sant'Ana, & M. Koubaa (Eds.), *Innovative technologies for food preservation: Inactivation of spoilage and pathogenic microorganisms* (pp. 53–107.).Academic Press. https://doi.org/10.1016/B978-0-12-811031-7.00003-0

LOTFIPOUR, F., MIRZAEEI, S., & MAGHSOODI, M. (2012): Evaluation of the effect of cacl2 and alginate concentrations and hardening time on the characteristics of *Lactobacillus acidophilus* loaded alginate beads using response surface analysis. *Advanced Pharmaceutical Bulletin*, *2*(1), 71–78. https://doi.org/10.5681/apb.2012.010

LV, T., YE, M., LUO, F., HU, B., WANG, A., CHEN, J., YAN, J., HE, Z., CHEN, F., QIAN, C., ZHANG, J., LIU, Z., & DING, Z. (2021): Probiotics treatment improves cognitive impairment in patients and animals: A systematic review and meta-analysis. *Neuroscience & Biobehavioral Reviews*, *120*, 159–172. https://doi.org/10.1016/j.neubiorev.2020.10.027

M. WAYS, T., LAU, W., & KHUTORYANSKIY, V. (2018): Chitosan and Its Derivatives for Application in Mucoadhesive Drug Delivery Systems. *Polymers*, *10*(3), 267. https://doi.org/10.3390/polym10030267

MAHARAJ, L. L., GUPTA, M. M., & GADAD, A. K. (2015): Microsphere : a Novel Tool for Controlled Drug. *World Journal of Pharmacy and Pharmaceutical Sciences*, *4*(09), 126–141.

MAHMOUD, M., ABDALLAH, N. A., EL-SHAFEI, K., TAWFIK, N. F., & EL-SAYED, H. S. (2020): Survivability of alginate-microencapsulated *Lactobacillus plantarum* during storage, simulated food processing and gastrointestinal conditions. *Heliyon*, *6*(3). https://doi.org/10.1016/j.heliyon.2020.e03541

MAJZOOBI, M., & BEPARVA, P. (2014): Effects of acetic acid and lactic acid on physicochemical characteristics of native and cross-linked wheat starches. *Food Chemistry*, *147*, 312–317. https://doi.org/10.1016/j.foodchem.2013.09.148

MALEKJANI, N., & JAFARI, S. M. (2020): Modeling the release of food bioactive ingredients from carriers/nanocarriers by the empirical, semiempirical, and mechanistic models. *Comprehensive Reviews in Food Science and Food Safety*, 20 (1), 3–47. Blackwell Publishing Inc. https://doi.org/10.1111/1541-4337.12660

MANIA, S. (2013): Microcapsules and their applications in pharmaceutical and food industry. *PhD Interdisciplinary Journal*, *2*(2), 71–75.

MARMOUZI, I., & EZZAT, S. M. (2018): The pharmacology of avenanthramides: Polyphenols. In: R. R. Watson, V. R. Preedy, & S. Zibadi (Eds.), *Polyphenols: Prevention and Treatment of Human Disease*. https://doi.org/10.1016/B978-0-12-813008-7.00001-1

MARTIN, M. J., LARA-VILLOSLADA, F., RUIZ, M. A., & MORALES, M. E. (2013): Effect of unmodified starch on viability of alginate-encapsulated *Lactobacillus fermentum* CECT5716. *LWT - Food Science and Technology*, *53*(2), 480–486. https://doi.org/zou 2011

MARTÍN, M. J., LARA-VILLOSLADA, F., RUIZ, M. A., & MORALES, M. E. (2015): Microencapsulation of bacteria: A review of different technologies and their impact on the probiotic effects. *Innovative Food Science and Emerging Technologies*, *27*, 15–25. https://doi.org/10.1016/j.ifset.2014.09.010

MARTINEZ, R. C. R., BEDANI, R., & SAAD, S. M. I. (2015): Scientific evidence for health effects attributed to the consumption of probiotics and prebiotics: an update for current perspectives and future challenges. *British Journal of Nutrition*, *114*(12), 1993–2015. https://doi.org/10.1017/S0007114515003864

MARTINS, E. M. F., RAMOS, A. M., VANZELA, E. S. L., STRINGHETA, P. C., DE OLIVEIRA PINTO, C. L., & MARTINS, J. M. (2013): Products of vegetable origin: A new alternative for the consumption of probiotic bacteria. *Food Research International*, *51*(2), 764–770. https://doi.org/10.1016/j.foodres.2013.01.047

MATTARELLI, P., & BIAVATI, B. (2018): Species in the Genus Bifidobacterium. In: P. Mattarelli, B. Biavati, W. H. Holzapfel, & B. J. B. Wood (Eds.), *The Bifidobacteria and Related Organisms* (pp. 9–48.).Academic Press. https://doi.org/10.1016/b978-0-12-805060-6.00002-8

MAZLOOM, K., SIDDIQI, I., & COVASA, M. (2019): Probiotics: How effective are they in the fight against obesity? *Nutrients*, *11*(2), 1–24. https://doi.org/10.3390/nu11020258

MCMASTER, L. D., KOKOTT, S. A., & SLATTER, P. (2005): Micro-encapsulation of *Bifidobacterium lactis* for incorporation into soft foods. *World Journal of Microbiology and Biotechnology*. https://doi.org/10.1007/s11274-004-4798-0

MGAYA-KILIMA, B., REMBERG, S. F., CHOVE, B. E., & WICKLUND, T. (2014): Influence of storage temperature and time on the physicochemical and bioactive properties of roselle-fruit juice blends in plastic bottle. *Food Science & Nutrition*, *2*(2), 181–191. https://doi.org/10.1002/fsn3.97

MILES, A. A., MISRA, S. S., & IRWIN, J. O. (1938): The estimation of the bactericidal power of the blood. *Journal of Hygiene*, *38*(6), 732–749. https://doi.org/10.1017/S002217240001158X

MINEKUS, M., ALMINGER, M., ALVITO, P., BALLANCE, S., BOHN, T., BOURLIEU, C., CARRIÈRE, F., BOUTROU, R., CORREDIG, M., DUPONT, D., DUFOUR, C., EGGER, L., GOLDING, M., KARAKAYA, S., KIRKHUS, B., LE FEUNTEUN, S., LESMES, U., MACIERZANKA, A., MACKIE, A., … BRODKORB, A. (2014): A standardised static *in vitro* digestion method suitable for food - an international consensus. *Food & Function*, *5*(6), 1113–1124. https://doi.org/10.1039/c3fo60702j

MINELLI, E. B., & BENINI, A. (2008): Relationship between number of bacteria and their probiotic effects. *Microbial Ecology in Health and Disease*, *20*(4), 180–183. https://doi.org/10.1080/08910600802408095

MIRMIRAN, P., HOUSHIALSADAT, Z., GAEINI, Z., BAHADORAN, Z., & AZIZI, F. (2020): Functional properties of beetroot (Beta vulgaris) in management of cardio-metabolic diseases. *Nutrition & Metabolism*, *17*(1), 3. https://doi.org/10.1186/s12986-019-0421-0

MISHRA, M. K. (2015a): Overview of Encapsulation and Controlled Release. In: M. Mishra (Ed.), *Handbook of Encapsulation and Controlled Release* (1st ed., pp. 3–19.).CRC Press. https://doi.org/10.1201/b19038-3

MISHRA, M. K. (2015b): Materials of Natural Origin for Encapsulation. In: M. K. Mishra (Ed.), *Handbook of Encapsulation and Controlled Release* (1st ed., pp. 517–540.).CRC Press. https://doi.org/10.1201/b19038-34

MU, W., CHEN, Q., WANG, X., ZHANG, T., & JIANG, B. (2013): Current studies on physiological functions and biological production of lactosucrose. *Applied Microbiology and Biotechnology*, *97*(16), 7073–7080. https://doi.org/10.1007/s00253-013-5079-3

MURIANA, P. M., & LUCHANSKY, J. B. (1993): Biochemical Methods for Purification of Bacteriocins. In: Dallas G. Hoover & L. R. Steenson (Eds.), *Bacteriocins of Lactic Acid Bacteria* (pp. 41–61.).Elsevier. https://doi.org/10.1016/B978-0-12-355510-6.50011-7

MUTHUKUMARASAMY, P., ALLAN-WOJTAS, P., & HOLLEY, R. A. (2006): Stability of *Lactobacillus reuteri* in different types of microcapsules. *Journal of Food Science*, *71*(1), M20–M24. https://doi.org/10.1111/j.1365-2621.2006.tb12395.x

MY DONG, L. (2020): The viability of encapsulated *Lactobacillus plantarum* during cupcake baking process, storage, and simulated gastric digestion. *Journal of Microbiology, Biotechnology and Food Sciences*, *9*(6), 1157–1161. https://doi.org/10.15414/jmbfs.2020.9.6.1157-1161

MY DONG, L., THI HANH QUYEN, L., DUC THANG, T., & THI KIM THUY, D. (2020): The Effects of Extrusion and Internal Emulsion Microencapsulation Methods on the Viability of *Lactobacillus*

acidophilus. *Journal of Human, Environment, and Health Promotion*, *6*(1), 1–5. https://doi.org/10.29252/jhehp.6.1.1

NAG, A. (2011)*: Development of a microencapsulation technique for probiotic bacteria Lactobacillus casei 431 using a protein-polysaccharide complex.* [Massey University]. http://mro.massey.ac.nz/handle/10179/2355

NAKAZAWA, Y., & HOSONO, A. (1992)*: Functions of Fermented Milk.*Elsevier Applied Science.

NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION (2020)*PubChem Compound Summary*PubChem. https://pubchem.ncbi.nlm.nih.gov/compound

NEUBAIER, H., & MOLLET, B. (2002): Biotechnological Research and the Dairy Industry. In: M. Hofman & P. Thonart (Eds.), *Engineering and manufacturing for biotechnology* (4th ed., pp. 399–412.).Kluwer Academic Publishers. https://doi.org/10.1007/0-306-46889-1_27

NO, H. K., YOUNG PARK, N., HO LEE, S., & MEYERS, S. P. (2002): Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. *International Journal of Food Microbiology*, *74*(1–2), 65–72. https://doi.org/10.1016/S0168-1605(01)00717-6

NUALKAEKUL, S., LENTON, D., COOK, M. T., KHUTORYANSKIY, V. V., & CHARALAMPOPOULOS, D. (2012): Chitosan coated alginate beads for the survival of microencapsulated *Lactobacillus plantarum* in pomegranate juice. *Carbohydrate Polymers*, *90*(3), 1281–1287. https://doi.org/10.1016/j.carbpol.2012.06.073

NURUNNABI, M., REVURI, V., HUH, K. M., & LEE, Y. KYU (2017): Polysaccharide based nano/microformulation: An effective and versatile oral drug delivery system. In: E. Andronescu & A. M. Grumezescu (Eds.), *Nanostructures for Oral Medicine* (pp. 409–433.).Elsevier. https://doi.org/10.1016/B978-0-323-47720-8.00015-8

O'MALLEY, J. M., & ZIESSMAN, H. (2014): Gastrointestinal System. In: H. A. Ziessman, J. P. O'Malley, & J. H. Thrall (Eds.), *Nuclear Medicine* (4th ed., pp. 288–321.).Elsevier. https://doi.org/10.1016/B978-0-323-08299-0.00013-4

O'RIORDAN, K., ANDREWS, D., BUCKLE, K., & CONWAY, P. (2001): Evaluation of microencapsulation of a *Bifidobacterium* strain with starch as an approach to prolonging viability during storage. *Journal of Applied Microbiology*. https://doi.org/10.1046/j.1365-2672.2001.01472.x

O'SULLIVAN, D. J., LEE, J. H., & DOMINGUEZ, W. (2011): Lactic Acid Bacteria: Genomics, Genetic Engineering. In: J. W. Fuquay (Ed.), *Encyclopedia of Dairy Sciences: Second Edition* (pp. 67–77.).Academic Press. https://doi.org/10.1016/B978-0-12-374407-4.00514-8

OKURO, P. K., THOMAZINI, M., BALIEIRO, J. C. C., LIBERAL, R. D. C. O., & FÁVARO-TRINDADE, C. S. (2013): Co- encapsulation of *Lactobacillus acidophilus* with inulin or polydextrose in solid lipid microparticles provides protection and improves stability. *Food Research International*, *53*(1), 96–103. https://doi.org/10.1016/j.foodres.2013.03.042

OLIVARES, A., SOTO, C., CABALLERO, E., & ALTAMIRANO, C. (2019): Survival of microencapsulated *Lactobacillus casei* (prepared by vibration technology) in fruit juice during cold storage. *Electronic Journal of Biotechnology*, *42*, 42–48. https://doi.org/10.1016/j.ejbt.2019.10.002

ÖNNING, G., ÅKESSON, B., ÖSTE, R., & LUNDQUIST, I. (1998): Effects of consumption of oat milk, soya milk, or cow's milk on plasma lipids and antioxidative capacity in healthy subjects. *Annals of Nutrition and Metabolism*, *42*(4), 211–220. https://doi.org/10.1159/000012736

ONSOYEN, E. (1992): Alginates. In: A. Imeson (Ed.), *Thickening and Gelling Agents for Food* (pp. 1–24.).Springer, Boston, MA. https://doi.org/10.1007/978-1-4615-3552-2_1

OUELLETTE, V., CHEVALIER, P., & LACROIX, C. (1994): Continuous fermentation of a supplemented

milk with immobilized *Bifidobacterium infantis*. *Biotechnology Techniques*, *8*(1), 45–50. https://doi.org/10.1007/BF00207632

PANESAR, P. S., & BALI, V. (2016): Prebiotics. In: B. Caballero, P. M. Finglas, & F. Toldrá (Eds.), *Encyclopedia of Food and Health* (pp. 464–471.).Elsevier. https://doi.org/10.1016/B978-0-12-384947-2.00560-2

PATIL, J. S., MARAPUR, S. C., GURAV, P. B., & BANAGAR, A. V. (2015): Ionotropic Gelation and Polyelectrolyte Complexation Technique: Novel Approach to Drug Encapsulation. In: M. Mishra (Ed.), *Handbook of Encapsulation and Controlled Release* (1st ed., pp. 297–320.).CRC Press. https://doi.org/10.1201/b19038-21

PECH-CANUL, A. DE LA C., ORTEGA, D., GARCÍA-TRIANA, A., GONZÁLEZ-SILVA, N., & SOLIS-OVIEDO, R. L. (2020): A Brief Review of Edible Coating Materials for the Microencapsulation of Probiotics. *Coatings*, *10*(3), 197. https://doi.org/10.3390/coatings10030197

PENHASI, A. (2015): Microencapsulation of probiotic bacteria using thermo-sensitive sol-gel polymers for powdered infant formula. *Journal of Microencapsulation*, *32*(4), 372–380. https://doi.org/10.3109/02652048.2015.1028497

PICOT, A., & LACROIX, C. (2004): Encapsulation of bifidobacteria in whey protein-based microcapsules and survival in simulated gastrointestinal conditions and in yoghurt. *International Dairy Journal*, *14*(6), 505–515. https://doi.org/10.1016/j.idairyj.2003.10.008

PINTO, S. S., CAVALCANTE, B. D., VERRUCK, S., ALVES, L. F., PRUDÊNCIO, E. S., & AMBONI, R. D. (2017): Effect of the incorporation of *Bifidobacterium* Bb-12 microencapsulated with sweet whey and inulin on the properties of Greek-style yogurt. *Journal of Food Science and Technology*, *54*(9), 2804–2813. https://doi.org/10.1007/s13197-017-2717-2

PLAVEC, T. V., & BERLEC, A. (2020): Safety Aspects of Genetically Modified Lactic Acid Bacteria. *Microorganisms*, *8*(2), 297. https://doi.org/10.3390/microorganisms8020297

PLUMBRIDGE, J. (2009): Regulation of Carbon Assimilation in Bacteria. In: M. Schaechter (Ed.), *Encyclopedia of Microbiology* (3rd ed., pp. 375–394.).Elsevier. https://doi.org/10.1016/B978-012373944-5.00067-5

PONCELET, D. (2001): Production of alginate beads by emulsification/internal gelation. *Annals of the New York Academy of Sciences*, *944*(1), 74–82. https://doi.org/10.1111/j.1749-6632.2001.tb03824.x

PORFIRYEVA, N. N., NASIBULLIN, S. F., ABDULLINA, S. G., TUKHBATULLINA, I. K., MOUSTAFINE, R. I., & KHUTORYANSKIY, V. V. (2019): Acrylated Eudragit® E PO as a novel polymeric excipient with enhanced mucoadhesive properties for application in nasal drug delivery. *International Journal of Pharmaceutics*, *562*(March), 241–248. https://doi.org/10.1016/j.ijpharm.2019.03.027

PRIYA, A. J., VIJAYALAKSHMI, S. P., & RAICHUR, A. M. (2011): Enhanced survival of probiotic *Lactobacillus acidophilus* by encapsulation with nanostructured polyelectrolyte layers through layer-by-layer approach. *Journal of Agricultural and Food Chemistry*, *59*(21), 11838–11845. https://doi.org/10.1021/jf203378s

PROBIOTICS MARKET (2019)*Probiotics Market Trends, Size, Analysis*. https://www.marketsandmarkets.com/Market-Reports/probiotic-market-advanced-technologies-and-global-market-69.html

PURDIE, J., & SYN, M. (2020, January 18): Oat Milk Nutrition Facts. *Verywell Fit*. https://www.verywellfit.com/oat-milk-nutrition-facts-4771939

PYCLIK, M., SRUTKOVA, D., SCHWARZER, M., & GÓRSKA, S. (2020): Bifidobacteria cell wall-derived

exo-polysaccharides, lipoteichoic acids, peptidoglycans, polar lipids and proteins – their chemical structure and biological attributes. *International Journal of Biological Macromolecules*, *147*, 333–349. https://doi.org/10.1016/j.ijbiomac.2019.12.227

QUIGLEY, E. M. M. (2017): *Bifidobacterium animalis spp. lactis*. In: M. H. Floch, Y. Ringel, & W. A. Walker (Eds.), *The Microbiota in Gastrointestinal Pathophysiology: Implications for Human Health, Prebiotics, Probiotics, and Dysbiosis* (pp. 127–130.).Academic Press. https://doi.org/10.1016/B978-0-12-804024-9.00013-6

QUIROZ-ITURRA, L., ROSAS-SAAVEDRA, C., & STANGE KLEIN, C. (2017): Functional Fruits Through Metabolic Engineering. *Superfood and Functional Food - The Development of Superfoods and Their Roles as Medicine*, 47. https://doi.org/10.5772/67219

RAHILA PARVEEN, S., & PREETHA, R. (2020): Study on Colour Stability and Microencapsulation of Anthocyanin Pigment Extracted from Dragon Fruit Peel by Spray and Freeze Drying. *Proceedings International*, *2*(1), 47. https://doi.org/10.33263/Proceedings21.047047

RAJAM, R., KUMAR, S. B., PRABHASANKAR, P., & ANANDHARAMAKRISHNAN, C. (2015): Microencapsulation of *Lactobacillus plantarum* MTCC 5422 in fructooligosaccharide and whey protein wall systems and its impact on noodle quality. *Journal of Food Science and Technology*, *52*(7), 4029–4041. https://doi.org/10.1007/s13197-014-1506-4

RAO, A. V., SHIWNARAIN, N., & MAHARAJ, I. (1989): Survival of Microencapsulated *Bifidobacterium pseudolongum* in Simulated Gastric and Intestinal Juices. *Canadian Institute of Food Science and Technology Journal*, *22*(4), 345–349. https://doi.org/10.1016/s0315-5463(89)70426-0

RAZAVI, S. M. A., & KARAZHIYAN, H. (2012): Rheological and textural characteristics of date paste. *International Journal of Food Properties*, *15*(2), 281–291. https://doi.org/10.1080/10942912.2010.483615

REID, A. A., CHAMPAGNE, C. P., GARDNER, N., FUSTIER, P., & VUILLEMARD, J. C. (2007): Survival in food systems of *Lactobacillus rhamnosus* R011 microentrapped in whey protein gel particles. *Journal of Food Science*, *72*(1), M031–M037. https://doi.org/10.1111/j.1750-3841.2006.00222.x

REIS, C. P., NEUFELD, R. J., VILELA, S., RIBEIRO, A. J., & VEIGA, F. (2006): Review and current status of emulsion/dispersion technology using an internal gelation process for the design of alginate particles. *Journal of Microencapsulation*, *23*(3), 245–257. https://doi.org/10.1080/02652040500286086

RESCH-GENGER, U. (2008): Standardization and Quality Assurance in Fluorescence Measurements II. In: U. Resch-Genger (Ed.), *Standardization and Quality Assurance in Fluorescence Measurements II* (Vol. 6).Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-540-70571-0

RIAZ, Q. U. A., & MASUD, T. (2013): Recent Trends and Applications of Encapsulating Materials for Probiotic Stability. *Critical Reviews in Food Science and Nutrition*, *53*(3), 231–244. https://doi.org/10.1080/10408398.2010.524953

RIUS, N., SOLÃ©, M., FRANCIA, A., & LORÃ©N, J.-G. (1994): Buffering capacity and membrane H + conductance of lactic acid bacteria. *FEMS Microbiology Letters*, *120*(3), 291–295. https://doi.org/10.1111/j.1574-6968.1994.tb07048.x

RIVERA-ESPINOZA, Y., & GALLARDO-NAVARRO, Y. (2010): Non-dairy probiotic products. *Food Microbiology*, *27*(1), 1–11. https://doi.org/10.1016/j.fm.2008.06.008

ROBERFROID, M. B. (2000): A European consensus of scientific concepts of functional foods.

Nutrition, *16*(7–8), 689–691. https://doi.org/10.1016/S0899-9007(00)00329-4

ROY, D. (2019): Probiotics. In: M. Moo-Young (Ed.), *Comprehensive Biotechnology* (3rd ed., Vol. 4, pp. 649–661.).Elsevier. https://doi.org/10.1016/B978-0-444-64046-8.00245-7

RUIZ, L., GUEIMONDE, M., RUAS-MADIEDO, P., RIBBERA, A., DE LOS REYES-GAVILÁN, C. G., VENTURA, M., MARGOLLES, A., & SÁNCHEZ, B. (2012): Molecular Clues To Understand the Aerotolerance Phenotype of *Bifidobacterium animalis subsp. lactis*. *Applied and Environmental Microbiology*, *78*(3), 644–650. https://doi.org/10.1128/AEM.05455-11

SAARELA, M. (2017): Future Development of Probiotic Dairy Products. In: Adnan Y. Tamime & L. V. Thomas (Eds.), *Probiotic Dairy Products* (2nd ed., pp. 389–394.).John Wiley & Sons, Ltd. https://doi.org/10.1002/9781119214137.ch10

SABIKHI, L., SUTHAR, B., & THOMPKINSON, D. (2011): Co-encapsulation with inulin to improve the survival of probiotic *Lactobacillus acidophilus* NCDC15 for synbiotic food applications. *Milchwissenschaft*, *66*, 18–22.

SALLEHUDIN, N. S., KHALIL, K. A., MUSA, M., YAZID, H. N. M., & YUSOF, A. (2017): Release Activity of Encapsulated *Lactobacillus plantarum* NBRC 3070 in Optimum Alginate - *Aloe vera* Matrices during Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) Exposure. *Asian Journal of Applied Sciences*, *5*(2), 383–388. https://doi.org/10.24203/ajas.v5i2.4500

SAMEDI, L., & CHARLES, A. L. (2019): Viability of 4 probiotic bacteria microencapsulated with arrowroot starch in the simulated gastrointestinal tract (GIT) and yoghurt. *Foods*, *8*(5). https://doi.org/10.3390/foods8050175

SANDERS, E. R. (2012): Aseptic laboratory techniques: plating methods. *Journal of Visualized Experiments : JoVE*, *63*, e3064. https://doi.org/10.3791/3064

SANDOVAL-CASTILLA, O., LOBATO-CALLEROS, C., GARCÍA-GALINDO, H. S., ALVAREZ-RAMÍREZ, J., & VERNON-CARTER, E. J. (2010): Textural properties of alginate-pectin beads and survivability of entrapped Lb. casei in simulated gastrointestinal conditions and in yoghurt. *Food Research International*. https://doi.org/10.1016/j.foodres.2009.09.010

SARAO, L. K., & ARORA, M. (2017): Probiotics, prebiotics, and microencapsulation: A review. *Critical Reviews in Food Science and Nutrition*, *57*(2), 344–371. https://doi.org/10.1080/10408398.2014.887055

SCARDOVI, V. (1981): The Genus Bifidobacterium. In: A. Parte, W. B. Whitman, M. Goodfellow, P. Kämpfer, H.-J. Busse, M. E. Trujillo, W. Ludwig, & K. Suzuki (Eds.), *Bergey's Manual of Systematic Bacteriology: The Actinobacteria* (2nd ed., pp. 1951–1961.).Springer-Verlag.

SCHOCH, C. L., CIUFO, S., DOMRACHEV, M., HOTTON, C. L., KANNAN, S., KHOVANSKAYA, R., LEIPE, D., MCVEIGH, R., O'NEILL, K., ROBBERTSE, B., SHARMA, S., SOUSSOV, V., SULLIVAN, J. P., SUN, L., TURNER, S., & KARSCH-MIZRACHI, I. (2020): NCBI Taxonomy: a comprehensive update on curation, resources and tools. *Database : The Journal of Biological Databases and Curation*, *2020*. https://doi.org/10.1093/database/baaa062

SENGSAENGTHONG, S., & OONSIVILAI, R. (2019): Effect of microencapsulation of *Lactobacillus* sp. 21C2-10 isolated from cassava pulp on physicochemical, sensorial and microbiological characteristics of ice cream. *International Food Research Journal*, *26*(2), 585–594.

SETHI, S., TYAGI, S. K., & ANURAG, R. K. (2016): Plant-based milk alternatives an emerging segment of functional beverages: a review. *Journal of Food Science and Technology*, *53*(9), 3408–3423. https://doi.org/10.1007/s13197-016-2328-3

SHAFIEI, Y. (2018): Probiotic and Synbiotic Yogurt Production Using Free or Alginate/Resistant

Starch Microencapsulated *Lactobacillus plantarum*. In: A.M. Grumezescu & A.M. Holban (Eds.), *Role of Materials Science in Food Bioengineering* (pp. 301–328.).Elsevier. https://doi.org/10.1016/B978-0-12-811448-3.00009-7

SHAH, N. P. (2011): BACTERIA, BENEFICIAL | *Bifidobacterium* spp.: Morphology and Physiology. In J. W. Fuquay (Ed.), *Encyclopedia of Dairy Sciences* (2nd ed., pp. 381–387.).Elsevier. https://doi.org/10.1016/B978-0-12-374407-4.00043-1

SHARIFI-RAD, J., RODRIGUES, C. F., STOJANOVIĆ-RADIĆ, Z., DIMITRIJEVIĆ, M., ALEKSIĆ, A., NEFFE-SKOCIŃSKA, K., ZIELIŃSKA, D., KOŁOŻYN-KRAJEWSKA, D., SALEHI, B., MILTON PRABU, S., SCHUTZ, F., DOCEA, A. O., MARTINS, N., & CALINA, D. (2020): Probiotics: Versatile Bioactive Components in Promoting Human Health. *Medicina*, *56*(9), 433. https://doi.org/10.3390/medicina56090433

SHARMA, A., GAUTAM, S., & WADHAWAN, S. (2014): Xanthomonas. In: C. A. Batt & M. Lou Tortorello (Eds.), *Encyclopedia of Food Microbiology* (pp. 811–817.).Elsevier. https://doi.org/10.1016/B978-0-12-384730-0.00359-1

SHEKHAR, K., MADHU, M. N., PRADEEP, B., & BANJI, D. (2010): A review on microencapsulation. *International Journal of Pharmaceutical Sciences Review and Research*, *5*(2), 58–62.

SHEU, T. Y., & MARSHALL, R. T. (1993): Microentrapment of Lactobacilli in Calcium Alginate Gels. *Journal of Food Science*, *58*(3), 557–561. https://doi.org/10.1111/j.1365-2621.1993.tb04323.x

SHILPI, S., & JAIN, S. K. (2016): Biodegradable Polymers: Drug Delivery Applications. In: M. Mishra (Ed.), *Handbook of Encapsulation and Controlled Release* (1st ed., pp. 646–670.).Taylor & Francis. https://doi.org/10.1081/E-EBPP-120049273

SIANG, S. C., WAI, L. K., LIN, N. K., & PHING, P. L. (2019): Effect of added prebiotic (Isomalto-oligosaccharide) and coating of beads on the survival of microencapsulated *Lactobacillus rhamnosus* GG. *Food Science and Technology*, *39*, 601–609. https://doi.org/10.1590/fst.27518

SIEGRIST, J. (2013)*: Lactobacilli.* https://www.sigmaaldrich.com/technical-documents/articles/microbiology-focus/lactobacilli.html

SILVÉRIO, S. C., MACEDO, E. A., TEIXEIRA, J. A., & RODRIGUES, L. R. (2015): Perspectives on the biotechnological production and potential applications of lactosucrose: A review. *Journal of Functional Foods*, *19*(Part A), 74–90. https://doi.org/10.1016/j.jff.2015.09.014

SIMPSON, P. J., STANTON, C., FITZGERALD, G. F., & ROSS, R. P. (2005): Intrinsic tolerance of *Bifidobacterium* species to heat and oxygen and survival following spray drying and storage. *Journal of Applied Microbiology*, *99*(3), 493–501. https://doi.org/10.1111/j.1365-2672.2005.02648.x

SINGH, M. N., HEMANT, K. S. Y., RAM, M., & SHIVAKUMAR, H. G. (2010): Microencapsulation: A promising technique for controlled drug delivery. *Research in Pharmaceutical Sciences*, *5*(2), 65–77. http://www.ncbi.nlm.nih.gov/pubmed/21589795

SINGH, P. K., DEOL, P. K., & KAUR, I. P. (2012): Entrapment of *Lactobacillus acidophilus* into alginate beads for the effective treatment of cold restraint stress induced gastric ulcer. *Food Funct.*, *3*(1), 83–90. https://doi.org/10.1039/C1FO10100E

SLAYTON, R., & PFAU, L. E. (2019)*List of Low Calorie Fruits & Vegetables*. https://www.livestrong.com/article/362699-list-of-low-calorie-fruits-vegetables/

ŚLIŻEWSKA, K., MARKOWIAK-KOPEĆ, P., & ŚLIŻEWSKA, W. (2020): The Role of Probiotics in Cancer Prevention. *Cancers*, *13*(1), 20. https://doi.org/10.3390/cancers13010020

SMIDSROD, O., & SKJAKBRK, G. (1990): Alginate as immobilization matrix for cells. *Trends in*

Biotechnology, *8*(C), 71–78. https://doi.org/10.1016/0167-7799(90)90139-O

SODINI, I., BOQUIEN, C. Y., CORRIEU, G., & LACROIX, C. (1997): Use of an immobilized cell bioreactor for the continuous inoculation of milk in fresh cheese manufacturing. *Journal of Industrial Microbiology and Biotechnology*, *18*(1), 56–61. https://doi.org/10.1038/sj.jim.2900362

SOLANKI, H. K., PAWAR, D. D., SHAH, D. A., PRAJAPATI, V. D., JANI, G. K., MULLA, A. M., & THAKAR, P. M. (2013): Development of microencapsulation delivery system for long-term preservation of probiotics as biotherapeutics agent. *BioMed Research International*, *2013*. https://doi.org/10.1155/2013/620719

SOLIMAN, A. H. S., SHAROBA, A. M., BAHLOL, H. E. M., SOLIMAN, A. S., & RADI, O. M. M. (2015): Evaluation of *Lactobacillus acidophilus*, *Lactobacillus casei* and *Lactobacillus plantarum* for probiotic characteristics. *Middle East Journal of Applied Sciences*, *05*(01), 10–18.

SOUSA E SILVA, J. P., & FREITAS, A. C. (2014)*: Probiotic bacteria: Fundamentals, therapy and technological aspects.* https://doi.org/10.4032/9789814411639

SUGASHINI, S., & GOPALAKRISHNAN, S. (2012): Studies on the Performance of Protonated cross linked Chitosan Beads (PCCB) for Chromium Removal. *Research Journal of Chemical Sciences*, *2*(6), 55–59.

SULTANA, K., GODWARD, G., REYNOLDS, N., ARUMUGASWAMY, R., PEIRIS, P., & KAILASAPATHY, K. (2000): Encapsulation of probiotic bacteria with alginate–starch and evaluation of survival in simulated gastrointestinal conditions and in yoghurt. *International Journal of Food Microbiology*, *62*(1–2), 47–55. https://doi.org/10.1016/S0168-1605(00)00380-9

SUN, W., & GRIFFITHS, M. W. (2000): Survival of bifidobacteria in yogurt and simulated gastric juice following immobilization in gellan–xanthan beads. *International Journal of Food Microbiology*, *61*(1), 17–25. https://doi.org/10.1016/S0168-1605(00)00327-5

SYNGAI, G. G., GOPI, R., BHARALI, R., DEY, S., LAKSHMANAN, G. M. A., & AHMED, G. (2016): Probiotics - the versatile functional food ingredients. *Journal of Food Science and Technology*, *53*(2), 921–933. https://doi.org/10.1007/s13197-015-2011-0

SZCZESNIAK, A. S. (2002): Texture is a sensory property. *Food Quality and Preference*, *13*(4), 215–225. https://doi.org/10.1016/S0950-3293(01)00039-8

SZILAGYI, A., & ISHAYEK, N. (2018): Lactose Intolerance, Dairy Avoidance, and Treatment Options. *Nutrients*, *10*(12), 1994. https://doi.org/10.3390/nu10121994

TABIKHA, M., EL-SHEHAWY, S., & HELAL, D. (2010): Changes in Chemical and Nutritional Quality During Cold Storage of Some Fruit and Vegetable Juice Blends. *Journal of Food and Dairy Sciences*, *1*(4), 181–191. https://doi.org/10.21608/jfds.2010.82107

TACER-CABA, Z., & NILUFER-ERDIL, D. (2019): Resistant Starch. In: L. Melton, F. Shahidi, & P. Varelis (Eds.), *Encyclopedia of Food Chemistry* (pp. 571–575.).Elsevier. https://doi.org/10.1016/B978-0-08-100596-5.22407-4

TALWALKAR, A., & KAILASAPATHY, K. (2004): The role of oxygen in the viability of probiotic bacteria with reference to *L. acidophilus* and *Bifidobacterium* spp. *Current Issues in Intestinal Microbiology*, *5*(1), 1–8. http://www.ncbi.nlm.nih.gov/pubmed/15055922

TAMIME, A. Y., & THOMAS, L. V. (2017): Probiotic Dairy Products. In: Adnan Y. Tamime & L. V. Thomas (Eds.), *Probiotic Dairy Products*.John Wiley & Sons, Ltd. https://doi.org/10.1002/9781119214137

TANIGUCHI, H., & HONNDA, Y. (2009): Amylases. In: M. Schaechter (Ed.), *Encyclopedia of Microbiology* (3rd ed., pp. 159–173.).Elsevier. https://doi.org/10.1016/B978-012373944-

5.00130-9

TEOH, P. L., MIRHOSSEINI, S. H., MUSTAFA, S., & MANAP, M. Y. A. (2011): Tolerance of free and encapsulated probiotics towards heat treatment and high sodium concentration. *Journal of Food, Agriculture and Environment*, *9*(1), 69–73. file:///paper/Tolerance-of-free-and-encapsulated-probiotics-heat-Peck-Mirhosseini/53ffcdd6d544c7d3d244ba8f050011e58d8d6e13

TERPOU, A., PAPADAKI, A., LAPPA, I., KACHRIMANIDOU, V., BOSNEA, L., & KOPSAHELIS, N. (2019): Probiotics in Food Systems: Significance and Emerging Strategies Towards Improved Viability and Delivery of Enhanced Beneficial Value. *Nutrients*, *11*(7), 1591. https://doi.org/10.3390/nu11071591

TOMASIK, P. (2003): Chemical and functional properties of food saccharides. In: P. Tomasik (Ed.), *Chemical and Functional Properties of Food Saccharides*.CRC Press. https://doi.org/10.1016/j.tifs.2004.03.002

TOMASIK, P., & HORTON, D. (2012): Enzymatic conversions of starch. In: D. Horton (Ed.), *Advances in Carbohydrate Chemistry and Biochemistry* (Vol. 68, pp. 59–436.).Academic Press Inc. https://doi.org/10.1016/B978-0-12-396523-3.00001-4

TORTAJADA-GENARO, L. A., SEITI, E., & MAQUIEIRA, Á. (2019): Talanta Consumer electronics devices for DNA genotyping based on loop-mediated isothermal amplification and array hybridisation. *Talanta*, *198*(February), 424–431. https://doi.org/10.1016/j.talanta.2019.01.124

TRIPATHI, M. K., & GIRI, S. K. (2014): Probiotic functional foods: Survival of probiotics during processing and storage. *Journal of Functional Foods*, *9*(1), 225–241. https://doi.org/10.1016/j.jff.2014.04.030

TUNGLAND, B. (2018): Overview of Prebiotics: Membership, Physiological Effects and their Health Attributes. In: B. Tungland (Ed.), *Human Microbiota in Health and Disease* (7th ed., pp. 289–348.).Elsevier. https://doi.org/10.1016/B978-0-12-814649-1.00007-7

VALERO-CASES, E., CERDÁ-BERNAD, D., PASTOR, J.-J., & FRUTOS, M.-J. (2020): Non-Dairy Fermented Beverages as Potential Carriers to Ensure Probiotics, Prebiotics, and Bioactive Compounds Arrival to the Gut and Their Health Benefits. *Nutrients*, *12*(6), 1666. https://doi.org/10.3390/nu12061666

VAN TASSELL, M. L., & MILLER, M. J. (2011): *Lactobacillus* adhesion to mucus. *Nutrients*, *3*(5), 613–636. https://doi.org/10.3390/nu3050613

VARUM, F. J. O., & BASIT, A. W. (2014): Gastrointestinal Mucosa and Mucus. In: Vitaliy V. Khutoryanskiy (Ed.), *Mucoadhesive Materials and Drug Delivery Systems* (Vol. 9781119941, pp. 83–98.).John Wiley & Sons, Ltd. https://doi.org/10.1002/9781118794203.ch04

VERHOECKX, K., COTTER, P., LÓPEZ-EXPÓSITO, I., KLEIVELAND, C., LEA, T., MACKIE, A., REQUENA, T., SWIATECKA, D., & WICHERS, H. (2015)*: The impact of food bioactives on health: In vitro and Ex Vivo models.* https://doi.org/10.1007/978-3-319-16104-4

VERSANTVOORT, C., KAMP, E. VAN DE, & ROMPELBERG, C. (2004): Development and applicability of an *in vitro* model in assessing the bioaccessibility of contaminants from food. In *RIVM report 320102002*.

VILLAMIEL, M., MONTILLA, A., OLANO, A., & CORZO, N. (2014): Production and Bioactivity of Oligosaccharides Derived from Lactose. In: F. J. Moreno & M. L. Sanz (Eds.), *Food Oligosaccharides: Production, Analysis and Bioactivity* (Vol. 9781118426, pp. 135–167.).John Wiley & Sons, Ltd. https://doi.org/10.1002/9781118817360.ch9

VINDEROLA, G., BINETTI, A., BURNS, P., & REINHEIMER, J. (2011): Cell Viability and Functionality of

Probiotic Bacteria in Dairy Products. *Frontiers in Microbiology*, *2*(MAY). https://doi.org/10.3389/fmicb.2011.00070

VINDEROLA, G., BURNS, P., & REINHEIMER, J. (2017): Probiotics in Nondairy Products. In: F. Mariotti (Ed.), *Vegetarian and Plant-Based Diets in Health and Disease Prevention* (pp. 809–835.).Elsevier. https://doi.org/10.1016/B978-0-12-803968-7.00044-7

VON WRIGHT, A., & AXELSSON, L. (2011): Lactic Acid Bacteria: An Introduction. In: S. Lahtinen, A. C. Ouwehand, S. Salminen, & A. von Wright (Eds.), *Lactic Acid Bacteria* (pp. 19–34.).CRC Press. https://doi.org/10.1201/b11503-5

WALLACE, T. C. (2015): Fiber Supplements and Clinically Meaningful Health Benefits: Identifying the Physiochemical Characteristics of Fiber that Drive Specific Physiologic Effects. In: T. C. Wallace (Ed.), *Dietary Supplements in Health Promotion* (1st ed., pp. 174–219.).CRC Press. https://doi.org/10.1201/b18490-12

WANDREY, C., BARTKOWIAK, A., & HARDING, S. E. (2010): Materials for Encapsulation. In: N. J. Zuidam & V. Nedovic (Eds.), *Encapsulation Technologies for Active Food Ingredients and Food Processing* (pp. 31–100.).Springer New York. https://doi.org/10.1007/978-1-4419-1008-0_3

WANG, H., LEE, I.-S., BRAUN, C., & ENCK, P. (2016): Effect of Probiotics on Central Nervous System Functions in Animals and Humans: A Systematic Review. *Journal of Neurogastroenterology and Motility*, *22*(4), 589–605. https://doi.org/10.5056/jnm16018

WANG, J., JANSEN, J. A., & YANG, F. (2019): Electrospraying: Possibilities and Challenges of Engineering Carriers for Biomedical Applications—A Mini Review. *Frontiers in Chemistry*, *7*, 258. https://doi.org/10.3389/fchem.2019.00258

WEN, J., CHEN, G., & ALANY, R. G. (2014): Theories and Concepts of Nano Materials, Nano- and microencapsulation. In: H.-S. Kwak (Ed.), *Nano- and Microencapsulation for Foods* (Vol. 9781118292, pp. 15–42.).John Wiley & Sons, Ltd. https://doi.org/10.1002/9781118292327.ch2

WHELEHAN, M., & MARISON, I. W. (2011): Microencapsulation using vibrating technology. *Journal of Microencapsulation*, *28*(8), 669–688. https://doi.org/10.3109/02652048.2011.586068

WHO/FAO (2001): Evaluation of health and nutritional properties of powder milk and live lactic acid bacteria. In *Food and Nutrition Paper*. https://doi.org/10.1201/9781420009613.ch16

WILLS, R. (2012): *In vitro* assessment of the survivability of *Lactobacillus casei* DN-114 001 and *Lactobacillus casei* Shirota, within commercialised food matrices, in the upper gastrointestinal tract. *The Plymouth Student Scientist*, *5*(2), 80–91.

WRUSS, J., WALDENBERGER, G., HUEMER, S., UYGUN, P., LANZERSTORFER, P., MÜLLER, U., HÖGLINGER, O., & WEGHUBER, J. (2015): Compositional characteristics of commercial beetroot products and beetroot juice prepared from seven beetroot varieties grown in Upper Austria. *Journal of Food Composition and Analysis*, *42*(3), 46–55. https://doi.org/10.1016/j.jfca.2015.03.005

XING, Y., XU, Q., MA, Y., CHE, Z., CAI, Y., & JIANG, L. (2014): Effect of porous starch concentrations on the microbiological characteristics of microencapsulated *Lactobacillus acidophilus*. *Food and Function*, *5*(5), 972–983. https://doi.org/10.1039/c3fo60438a

YANG, M., LIANG, Z., WANG, L., QI, M., LUO, Z., & LI, L. (2020): Microencapsulation Delivery System in Food Industry—Challenge and the Way Forward. *Advances in Polymer Technology*, *2020*, 1–14. https://doi.org/10.1155/2020/7531810

YAO, M., XIE, J., DU, H., MCCLEMENTS, D. J., XIAO, H., & LI, L. (2020): Progress in microencapsulation of probiotics: A review. *Comprehensive Reviews in Food Science and Food*

Safety, *19*(2), 857–874. https://doi.org/10.1111/1541-4337.12532

YE, Q., GEORGES, N., & SELOMULYA, C. (2018): Microencapsulation of active ingredients in functional foods: From research stage to commercial food products. *Trends in Food Science & Technology*, *78*, 167–179. https://doi.org/10.1016/j.tifs.2018.05.025

YEUNG, T. W., ÜÇOK, E. F., TIANI, K. A., MCCLEMENTS, D. J., & SELA, D. A. (2016): Microencapsulation in alginate and chitosan microgels to enhance viability of *Bifidobacterium longum* for oral delivery. *Frontiers in Microbiology*, *7*(APR), 494. https://doi.org/10.3389/fmicb.2016.00494

YING, D., SUN, J., SANGUANSRI, L., WEERAKKODY, R., & AUGUSTIN, M. A. (2012): Enhanced survival of spray-dried microencapsulated *Lactobacillus rhamnosus* GG in the presence of glucose. *Journal of Food Engineering*, *109*(3), 597–602. https://doi.org/10.1016/j.jfoodeng.2011.10.017

ZAEIM, D., SARABI-JAMAB, M., GHORANI, B., & KADKHODAEE, R. (2019): Double layer co-encapsulation of probiotics and prebiotics by electro-hydrodynamic atomization. *Lwt*, *110*(November 2018), 102–109. https://doi.org/10.1016/j.lwt.2019.04.040

ZAEIM, D., SARABI-JAMAB, M., GHORANI, B., KADKHODAEE, R., & TROMP, R. H. (2017): Electrospray assisted fabrication of hydrogel microcapsules by single- and double-stage procedures for encapsulation of probiotics. *Food and Bioproducts Processing*, *102*, 250–259. https://doi.org/10.1016/j.fbp.2017.01.004

ZAEIM, D., SARABI-JAMAB, M., GHORANI, B., KADKHODAEE, R., & TROMP, R. H. (2018): Electrospray-assisted drying of live probiotics in acacia gum microparticles matrix. *Carbohydrate Polymers*, *183*(December 2017), 183–191. https://doi.org/10.1016/j.carbpol.2017.12.001

ZAMAN, S. A., & SARBINI, S. R. (2015): The potential of resistant starch as a prebiotic. *Critical Reviews in Biotechnology*, *00*(00), 1–7. https://doi.org/10.3109/07388551.2014.993590

ZAMANI, H., DE JOODE, M. E. J. R., HOSSEIN, I. J., HENCKENS, N. F. T., GUGGEIS, M. A., BERENDS, J. E., DE KOK, T. M. C. M., & VAN BREDA, S. G. J. (2020): The benefits and risks of beetroot juice consumption: a systematic review. *Critical Reviews in Food Science and Nutrition*, *0*(0), 1–17. https://doi.org/10.1080/10408398.2020.1746629

ZHAO, M., WANG, Y., HUANG, X., GAENZLE, M., WU, Z., NISHINARI, K., YANG, N., & FANG, Y. (2018): Ambient storage of microencapsulated: *Lactobacillus plantarum* ST-III by complex coacervation of type-A gelatin and gum Arabic. *Food and Function*, *9*(2), 1000–1008. https://doi.org/10.1039/c7fo01802a

ZHENG, J., WITTOUCK, S., SALVETTI, E., FRANZ, C. M. A. P., HARRIS, H. M. B., MATTARELLI, P., O'TOOLE, P. W., POT, B., VANDAMME, P., WALTER, J., WATANABE, K., WUYTS, S., FELIS, G. E., GÄNZLE, M. G., & LEBEER, S. (2020): A taxonomic note on the genus Lactobacillus: Description of 23 novel genera, emended description of the genus Lactobacillus beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae. *International Journal of Systematic and Evolutionary Microbiology*, *70*(4), 2782–2858. https://doi.org/10.1099/ijsem.0.004107

ZHU, F. (2014): Influence of ingredients and chemical components on the quality of Chinese steamed bread. *Food Chemistry*, *163*, 154–162. https://doi.org/10.1016/j.foodchem.2014.04.067

ZIELIŃSKA, D., KOLOZYN-KRAJEWSKA, D., & LARANJO, M. (2018): Food-Origin Lactic Acid Bacteria May Exhibit Probiotic Properties: Review. *BioMed Research International*, *2018: 5063*, 1–15. https://doi.org/10.1155/2018/5063185

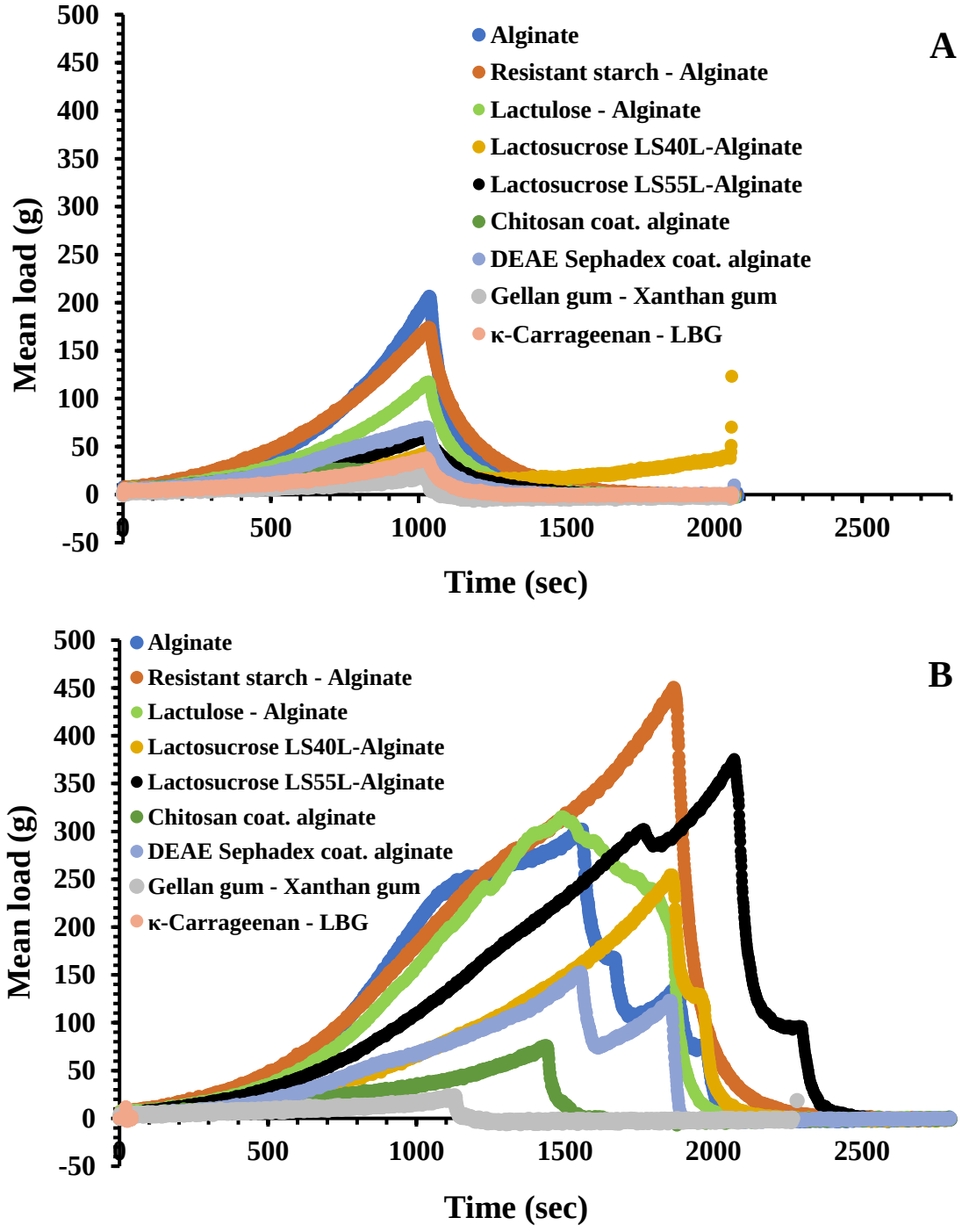
ZOU, Q., LIU, X., ZHAO, J., TIAN, F., ZHANG, H. P., ZHANG, H., & CHEN, W. (2012): Microencapsulation of *Bifidobacterium bifidum* F-35 in Whey Protein-Based Microcapsules by

Transglutaminase-Induced Gelation. *Journal of Food Science*, *77*(5), M270–M277. https://doi.org/10.1111/j.1750-3841.2012.02673.x

ZOU, Q., ZHAO, J., LIU, X., TIAN, F., ZHANG, H., ZHANG, H., & CHEN, W. (2011): Microencapsulation of *Bifidobacterium bifidum* F-35 in reinforced alginate microspheres prepared by emulsification/internal gelation. *International Journal of Food Science & Technology*, *46*(8), 1672–1678. https://doi.org/10.1111/j.1365-2621.2011.02685.x

ZOUMPOPOULOU, G., TSAKALIDOU, E., & THOMAS, L. V. (2018): An Overview of Probiotic Research: Human and Mechanistic Studies. In: A. Y. Thomas & T. L. V. (Eds.), *Probiotic Dairy Products* (2nd ed., pp. 293–357.).John Wiley & Sons, Ltd. https://doi.org/10.1002/9781119214137.ch8

FURTHER APPENDICES (A2.)



Appendix-Figure 1: Texture profile of each type of extrusion-formed gel capsules (based on 9 replicates on 10-capsule batch of each capsule type). A: Data obtained with non-destructive compression run; B: Data obtained with destructive compression run; LBG: locust bean gum.

Appendix-Figure 2: Brookfield LFRA 4500 Texture Analyzer

Appendix-Table 1. Frameset of parameters applied for simulating the oral, gastric and duodenum phases to perform the Infogest digestion protocol described by Minekus et al. (2014). Data highlighted in grey indicate all those parameters that varied among the different types of samples (i.e., free, alginate-, resistant starch-alginate-, chitosan coated alginate-encapsulated cells).

I. Oral phase

Duration: 2 min (the time amylase is active). Simulated salivary fluid = SSF.

Composition of SSF	**Ratio**	
Intial form of sample (food)	5.0	mL of PBS suspension
[5/4] stock SSF (containing only electrolytes)	4.0	mL
0.3M $CaCl_2$	25.0	µL
Amylase stock solution	0.5	mL
DW required to fill SSF up to 10.0 mL	0.475	mL
Final volume of SSF	**10.0**	**mL**

II. Gastric phase

Duration: 2 h. Simulated gastric fluid = SGF.

Free cells		
Composition of SGF	**Ratio**	
SSF-digested liquid sample (food)	10.00	mL
[5/4] stock SGF (containing only electrolytes)	8.00	mL
Pepsin stock solution	1.00	mL
0.3 M $CaCl_2$	5.0	µL
HCl to adjust pH 3.0 at 1 M	0.4000	mL
DW required to fill SGF up to 20.00 mL	0.5950	mL
Total volume to be removed for sampling during gastric stage (mL)	1.00	mL
Final volume of SGF	**19.00**	**mL**

Alginate encapsuled cells		
Composition of SGF	**Ratio**	
SSF-digested liquid sample (food)	10.00	mL
[5/4] stock SGF (containing only electrolytes)	8.00	mL

Pepsin solution	1.00	mL
0.3 M $CaCl_2$	5.0	μL
HCl to adjust pH 3.0 at 1 M	0.1610	mL
DW required to fill SGF up to 20.00 mL	0.8340	mL
Total volume to be removed for sampling during gastric stage (mL)	1.00	mL
Final volume of SGF	**19.00**	**mL**

Resistant starch-Alginate encapsulated cells		
Composition of SGF	**Ratio**	
SSF-digested liquid sample (food)	10.00	mL
[5/4] stock SGF (containing only electrolytes)	8.00	mL
Pepsin stock solution	1.00	mL
0.3 M $CaCl_2$	5.0	μL
HCl to adjust pH 3.0 at 1 M	0.2100	mL
DW required to fill SGF up to 20.00 mL	0.7850	mL
Total volume to be removed for sampling during gastric stage (mL)	1.00	mL
Final volume of SGF	**19.00**	**mL**

Chitosan coated alginate encapsuled cells		
Composition of SGF	**Ratio**	
SSF-digested liquid sample (food)	10.00	mL
[5/4] stock SGF (containing only electrolytes)	8.00	mL
Pepsin solution	1.00	mL
0.3 M $CaCl_2$	5.0	μL
HCl to adjust pH 3.0 at 1 M	0.3100	mL
DW required to fill SGF up to 20.00 mL	0.6850	mL
Total volume to be removed for sampling	1.00	mL

during gastric stage (mL)		
Final volume of SGF	**19.00**	**mL**

III. Duodenum phase

Duration: 2 h. Simulated intestinal fluid = SIF.

Composition of SIF	**Ratio**	
SGF-digested liquid sample (food)	19.00	mL
[5/4] stock SIF (containing only electrolytes)	4.78	mL
Pancreatin **(in SIF)**	5.00	mL
Bile **(in SIF)**	5.42	mL
0.3 M $CaCl_2$	38.0	μL
1M NaOH for adjusting pH 7.0	0.38	mL
DW required to fill SIF up to 38.00 mL	3.3820	mL
Final volume of SIF	**38.00**	**mL**

Composition of SIF	**Ratio**	
SGF-digested liquid sample (food)	19.00	mL
[5/4] stock SIF (containing only electrolytes)	4.78	mL
Pancreatin **(in SIF)**	5.00	mL
Bile **(in SIF)**	5.42	mL
0.3 M $CaCl_2$	38.0	μL
1M NaOH for adjusting pH 7.0	0.0970	mL
DW required to fill SIF up to 38.00 mL	3.6650	mL
Final volume of SIF	**38.00**	**mL**

Resistant starch-Alginate encapsulated cells		
Composition of SIF	**Ratio**	
SGF-digested liquid sample (food)	19.00	mL
[5/4] stock SIF (containing only electrolytes)	4.78	mL
Pancreatin **(in SIF)**	5.00	mL
Bile **(in SIF)**	5.42	mL
0.3 M $CaCl_2$	38.0	μL
1 M NaOH for adjusting pH 7.0	0.0960	mL
DW required to fill SIF up to 38.00 mL	3.6660	mL
Final volume of SIF	**38.00**	**mL**

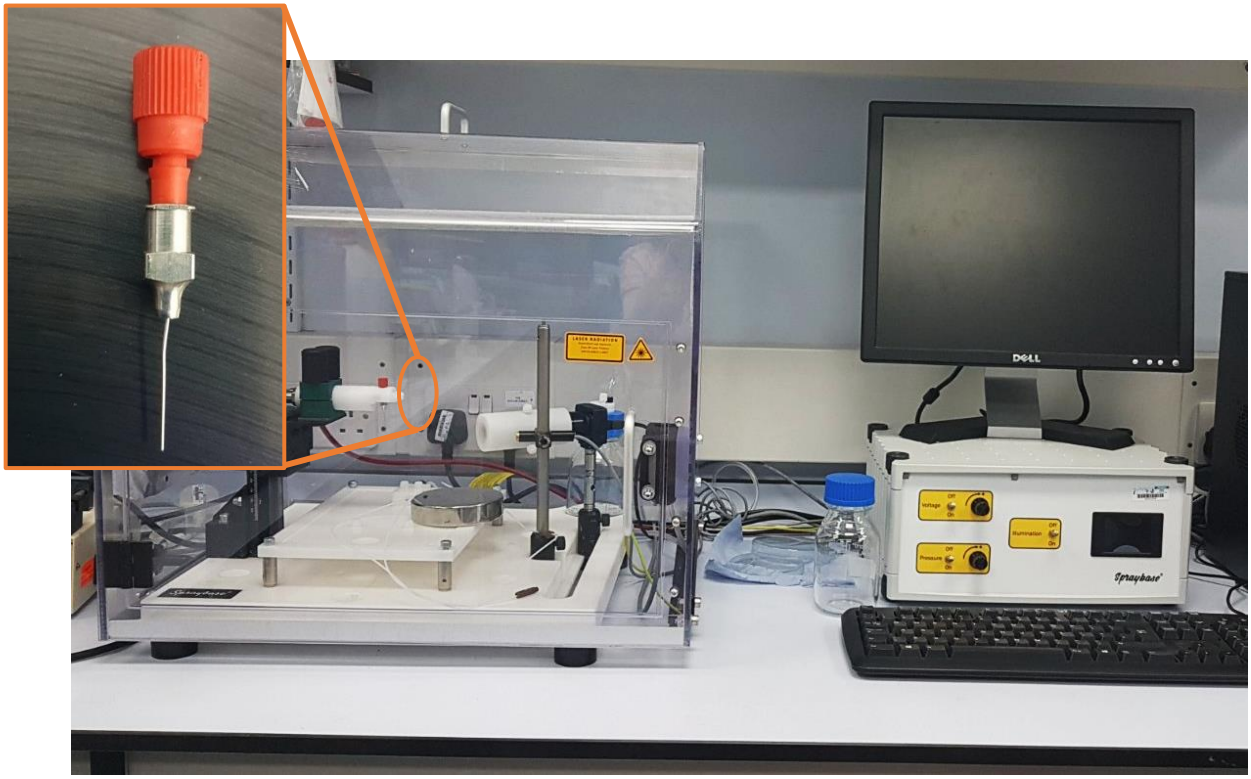
Chitosan coated alginate encapsuled cells		
Composition of SIF	**Ratio**	
SGF-digested liquid sample (food)	19.00	mL
[5/4] stock SIF (containing only electrolytes)	4.78	mL
Pancreatin **(in SIF)**	5.00	mL
Bile **(in SIF)**	5.42	mL
0.3 M $CaCl_2$	38.0	μL
1 M NaOH for adjusting pH 7.0	0.2850	mL
DW required to fill SIF up to 38.00 mL	3.4770	mL
Final volume of SIF	**38.00**	**mL**

Appendix-Table 2: Prepared stock solutions of different simulated digestion fluids applied in the *in vitro* Infogest digestion study. Table is an excerpt from the paper of Minekus et al. (2014).

			SSF		SGF		SIF	
			pH 7		pH 3		pH 7	
Constituent	Stock conc.		Vol. of stock	Conc. in SSF	Vol. of stock	Conc. in SGF	Vol. of stock	Conc. in SIF
	$g\ L^{-1}$	$mol\ L^{-1}$	mL	$mmol\ L^{-1}$	mL	$mmol\ L^{-1}$	mL	$mmol\ L^{-1}$
KCl	37.3	0.5	15.1	15.1	6.9	6.9	6.8	6.8
KH_2PO_4	68	0.5	3.7	3.7	0.9	0.9	0.8	0.8
$NaHCO_3$	84	1	6.8	13.6	12.5	25	42.5	85
NaCl	117	2	—	—	11.8	47.2	9.6	38.4
$MgCl_2(H_2O)_6$	30.5	0.15	0.5	0.15	0.4	0.1	1.1	0.33
$(NH_4)_2CO_3$	48	0.5	0.06	0.06	0.5	0.5	—	—
For pH adjustment								
	$mol\ L^{-1}$		mL	$mmol\ L^{-1}$	mL	$mmol\ L^{-1}$	mL	$mmol\ L^{-1}$
NaOH	1		—	—	—	—	—	—
HCl	6		0.09	1.1	1.3	15.6	0.7	8.4
$CaCl_2(H_2O)_2$ is not added to the simulated digestion fluids, see details in legend								
	$g\ L^{-1}$	$mol\ L^{-1}$		$mmol\ L^{-1}$		$mmol\ L^{-1}$		$mmol\ L^{-1}$
$CaCl_2(H_2O)_2$	44.1	0.3		1.5 (0.75*)		0.15 (0.075*)		0.6 (0.3*)

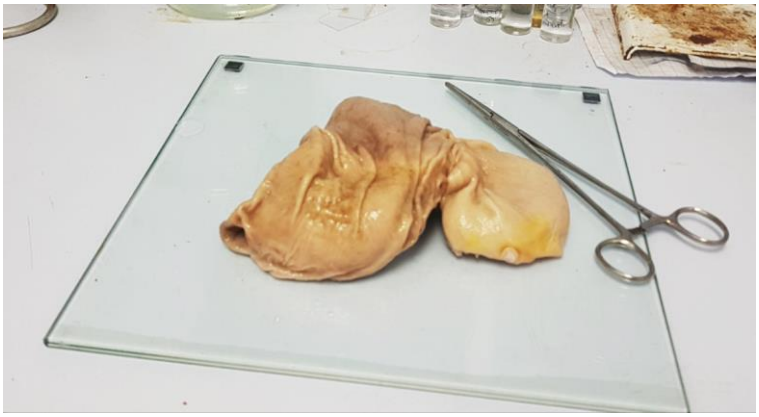
[a] * in brackets is the corresponding Ca^{2+} concentration in the final digestion mixture.

Appendix-Figure 3: Extrusion-formed (probiotic-loaded) gel capsules in oat drink after 5 months of storage at 20°C



Appendix-Figure 4: Applied electrospraying apparatus (Spraybase®, Avectas Ltd.) and the appearance of blunt needle emitter.

Appendix-Figure 5: 1080P 1000X Zoom HD 8LED Digital USB Microscope Magnifier Endoscope (left) and Winzwon UV Torch (right) used for the retention (mucoadhesion) study.



Appendix-Figure 6: An example of the porcine stomach before dissecting the mucosal tissues from it

.

ACKNOWLEDGEMENT

First and foremost, I would like to thank my supervisors, **Dr. Erika Bujna** and **Dr. Szilárd Kun** for all the patient guidance, valuable knowledge, and tremendous support they dedicated to my PhD research, to the publication of my scientific paper and to this thesis. Without their valuable supervision, the quality of my research work would have never been brought to a higher level.

I would like to express my gratitude to **Prof. Vitaliy V. Khutoryanskiy** for giving me a huge opportunity to work in his laboratory at the University of Reading for several months, for providing me an excellent supervision during this whole time, and for everything I learned from him about mucoadhesion and controlled drug delivery. Further, I also wish to thank him for financing all the tools and chemicals that I needed to complete my research work there, and also for giving me a chance to attend and present my research work at the conference of Innovation in Encapsulation in London. I am also very thankful to my second UK-based advisor, **Prof. Dimitris Charalampopoulos** for letting me pursue the microbiology-related part of my research work in his laboratory, and also for his valuable guidance and advice in this regard. Finally, I wish to thank both of them for offering me a precious opportunity to write and have a joint peer-reviewed scientific paper published with them.

The **European Union's Erasmus+** programme is also greatly thanked for funding my stay in Reading, UK.

Doctoral School of Food Science as well as the Foundation of **New Széchenyi Plant Project No. EFOP-3.6.3.-VEKOP-16-2017-00005** are also acknowledged for supporting my PhD studies and research.

I am very grateful for **Prof. Quang Duc Nguyen** for providing me his generous support, guidance, and encouragement during my PhD work and with the publication of my manuscript.

I am also highly grateful for **Dr. Otilia Tamara Antal**, from the Food Science Research Group, Institute of Food Science and Technology, Hungarian University of Agriculture and Life Sciences, for providing me a great opportunity to investigate my own probiotic formulations with Infogest digestion protocol, and also for generously supporting me in performing this particular experiment.

Furthermore, I also appreciate **Dr. Réka Juhász** formerly from the Department of Fruit and Vegetable Processing Technology for kindly allowing and helping me to use the texture analyser, and **Dr. Márta Ladányi** from the Department of Biometrics and Agricultural Informatics for her expert help in the statistical interpretation of my research data.

I would like to thank all of **my undergraduate and master's students** for their valuable contributions to my experiments.

I am also grateful for all the technical help and assistance from the **staff members** at the **Department of Bioengineering and Alcoholic Drink Technology of Hungarian University of Agriculture and Life Sciences,** from the **members** of the **Khutoryanskiy research group,** and from many of the **staff members** working at the **Department of Food and Nutritional Science, the University of Reading.**

Last but not least, I want to thank **my family**, who always supported me unconditionally and gave me the best possible backing even in difficult times.

Thank you all!