UNIVERSITY OF NAPLES "FEDERICO II"

DEPARTMENT OF AGRICULTURAL SCIENCES

AND

AN-NAJAH NATIONAL UNIVERSITY

FACULTY OF GRADUATE STUDIES

MASTER DEGREES IN

FOOD SCIENCE AND TECHNOLOGY

AND

NUTRITION AND FOOD TECHNOLOGY

Experimental Thesis

METABOLIC ATTENUATION OF *LACTICASEIBACILLUS CASEI* ATCC 393 USING AN EMULSION MICROENCAPSULATION STRATEGY

Tutor: Dr. Mohammad Altamimi **Candidate: HaneenAlrjoob**

Co-Tutor: Prof. MaurielloGianluigi **Instructor: Irene Giordano**

Academic year 2021-2022

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Academic year 2021-2022

Dedication

A special feeling of gratitude to my loving parents, Jamal and YusraRjoub whose words of encouragement and push for tenacity ring in my ears.

My sister and brothers Hanan, Hassan and Mohammad have never left my side and are very special.

I also dedicate this work to my beloved friends and guarding angel who have supported me throughout the process. I will always appreciate all they have done.

Acknowledgment

The big acknowledgment is for god for giving me the strength and patience to reach where I am.

A bit gratitude to who have taught me patiently my teachers and professors during the study journey from Al Najah National University to University of Naples Federico II,

Especially my instructor Irene Giordano for helping me develop my working skills, for teaching me generously, guiding me in the laboratory, and the many hours of proofreading.

Declaration

I, the undersigned, declare that I submitted the thesis entitled:

METABOLIC ATTENUATION OF *LACTICASEIBACILLUS CASEI* ATCC 393 USING AN EMULSION MICROENCAPSULATION STRATEGY

I declare that the work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

Student's Name: _____

Signature: _____

Date: _____

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METABOLIC ATTENUATION OF *LACTICASEIBACILLUS CASEI* ATCC 393 USING AN EMULSION MICROENCAPSULATION STRATEGY

By
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Supervisors
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ABSTRACT

Background: The attenuation of probiotic bacteria by microencapsulation can be considered as a physical approach to modulate the metabolic activities of the probiotic bacteria when inserted into the food matrix.

The aim: of this work is to study the attenuation effect of microencapsulation on *Lacticaseibacilluscasei* ATCC 393 fermentative metabolism. Then, the attenuating system developed was used to probioticate a food matrix, an orange juice, to test the efficacy of microencapsulation to avoid physico-chemical changes during the storage of the beverage.

Methodology: Two probiotic juices were formulated: LC_OJ added of the probiotic in free form and MC_OJ added of the probiotic as microcapsules of 0.8% sodium alginate coated with chitosan. Microbiological and physiochemical tests were carried out for the two juices during storage for 15 days at 4 °C and 15 °C.

Results: pH concluded that microencapsulation had no effect on pH when the juice is refrigerated. However the orange juice MC_OJ sample presented lower Titratable Acidity (TA) than LC_OJ for both storage temperatures, meaning that the microencapsulation lowered the TA. In this study, probiotication showed no effects on color change when the orange juice regardless the form of addition. The same is for the ascorbic acid content compared with the free cells form, due to the loss of the vitamin C content during samples preparations and storage. The concluded results for LC_OJ and MC_OJ, demonstrated that microencapsulation of *L. casei* ATCC 393 didn't have an attenuation effect. Both LC_OJ and MC_OJ presented high viable counts.

Conclusion: when the probiotic *L. casei* ATCC 393 is added to orange juice there is no need of attenuation. Moreover, orange juice represent a suitable matrix for the

probiotication maintaining constant chemical and physical parameters over time and simultaneously ensuring the survival of the probiotic.

Keywords: Attenuation, *L.casei* ATCC 393, Orange Juice, Viable counts, pH

Chapter One

Introduction

1.1 Theoretical basis and background

Food is considered to be an important and substantial element for the human life since the old times, because food supplies essential components for the physical, intellectual, and social development of humans. The quality of life is directly related to the diet and the general lifestyle. A well balanced diet can maintain a good health, moreover many studies showed the effectiveness of some foods in decreasing the risk of incidence of some diseases, and reducing the severity of the symptoms of some of them. Therefore, research and development of new natural ingredients has increased, especially for functional ingredients that have beneficial effects on health, such as probiotics microorganisms (1).

1.2 Probiotics

The term probiotics was first suggested in Greece in 1965 which means ‘for life’,. Originally, it was used to describe the secreted substances that stimulated the growth of organisms. In 2002 they had been defined by Marteau*et al.,* as the microbial preparations by the cells, in which they leave beneficial impacts on the human health. Whereas the Food and Agriculture organization (FAO), and the World Health Organization (WHO) have defined the probiotics as ‘live microorganisms which when administered in adequate amounts confer a health benefit on the host’(2). This definition was grammatically revised from Hill et al. (2014). These microorganisms can be found in fermented foods like fermented dairy products and vegetables, and could be presented by food supplements. Probiotics can be isolated from animal and human gastrointestinal systems, residual waters, stool, tooth decay and vagina (3). The most famous probiotics are bacteria, and they are the majority of the probiotics, the remained minority are distributed between yeast and moulds. The most well known and used probiotics bacteria belong to the genera of *Lactobacillus* and *Bifidobacterium.* In 2020 Zang et al. found four new genera from the genus *Lactobacillus.* These are *Lacticaseibacillus*, *Lactiplantibacillus*, *Ligilactobacillus* and *Limosilactobacillus*. Table1.1 shows a list of probiotics used for human and animal consumption.

Table 1.1

list of probiotics used for human and animal consumption(2)(4)(5).

Genus	Species
Lactobacillus	*rhamnosus, plantarum, casei, paracasei, reuteri, salivarius, acidophilus, plantarum, gasseri, leichmanii, jensenii, confusus, brevis, bulgaricus, lactis, fermentum, minutus,* and *catenaforme*sp.
Lactococcus	*Lactis*
Leuconostoc	*mesenteroides, paramesenteroides, citreum, pseudomesenteroides, and lactis sp.*
Pediococcus	*acidilactici and pentosaceus*
Bifidobacterium	*dentium (eriksonii),infantis,longum, thermophilum,bifidum,animalisadolescentis sp.*
Enterococcus	*faecalis, faecium, avium, and others*
Streptococcus	*thermophilus, thermophilus, salivarius subsp.*
Saccharomyces	*boulardii* *cerevisiae*
Others	*bacillus cereus, escherichia coli, enterococcus, propionibacterium* *freudenreichii*

Despite this long list, in reality they are considered as a very small part of the non-pathogenic microorganisms. It is important to note that the characteristics of probiotics are related to strain and not species. Therefore, the selection of the mentioned above probiotics was very specific, meaning that the isolation of the strains is by the approach of “step by step”, meaning that there are series of tests to gradually decrease the numbers of the microorganisms, and finally this process ends up with the selection of the strains that have the highest amounts of functional characteristics with viability, and at the same time, without bringing any negative features (3)(5).

1.2.1 Probiotics mechanism of action

Firstly, probiotics require to have a special stimulatory effect given by the prebiotics which are non-digestible elements of the food that usually come from the complex carbohydrates in vegetables, legumes, wheat bran, soybeans, grains, chicory etc…, aiding the probiotics to produce short chain fatty acids (SCFA). These non-digestible carbohydrates are fermented by *Lactobacilli*and *Bifidobacteria*spp. To have the optimum production of SCFA, prebiotics shouldn’t be digested in the upper gastrointestinal tract, and the microbiota should have the ability to ferment them, and to raise the abundance of the bacteria which is related to the health benefits of the host. Probiotics must survive gastric acidity and bile salts in the intestine, in addition to the ability to adhere to the epithelial cells and mucosa contributes to a longer retention into

the colon. Finally, the characteristics of the probiotics cells should be maintained stable during the processing of the certain type of food (6)(7).

1.2.2 Mechanism of action in exerting health benefits

- **Prevention of infectious diseases**

Probiotics contribute to the reduction of infections through several mechanisms. First of all,probiotics have the ability to inhibit pathogenic microorganisms competing with them for nutrient substrates. Plus, probiotics, by adhering to the intestinal walls bound the binding sites thus preventing the adhesion of pathogens. Moreover, by prebiotics fermentation, probiotics produce short chain fatty acids (SCFAs like butyric acid, lactic acid, and acetic acid) lowering the intestina pH; thus, creating an unfavorable environment for pathogenic microorganisms. Finally, they can produce antimicrobial metabolites like bacteriocins, and H_2O_2. An example on this type of probiotics is *Lactobacillus acidophilus* IBB 801,which was shown to yield two bacteriocins; the acidolin that inhibits the entero-pathogenic microbes, and the Lactacin B that inhibits the *Lactobacilli* in-vitro (8)(9).

- **Anti cancer activity**

Different ways have been investigated to chose the probiotics with the antitumor activity. Some pathogenic bacteria, like (some strains of *Escherichia coli, Bacteroides, Clostridium,* and*Eubacterium*) have the ability to activate some enzymes that produce carcinogenic compounds. Therefore, to inhibit and knock down the growth of these pathogenic microbes is a way by which probiotics can exert their anticancer activity. Other mechanisms involved binding and breaking down mutagens, production of substrates with anti-carcinogenic effects, reducing the effectiveness of some enzymes engaged in carcinogen building up, reduction of nephrotoxic mycotoxins and genotoxic immunosuppressive, blocking down the tumor cell proliferation, and finally inducing apoptosis in cancerous cells by the production of linoleic acid by the stains of *L. bulgaricus*and *S. thermophilus*(10).

- **Lowering Blood cholesterol**

Probiotics, have the ability to reduce the cholesterol solubility, therefore, they decrease its absorption by the gut, as a result, they are considered to have a prevention role among heart diseases such as the strain of *L. plantarum*PH04 (11). The lowering effect on cholesterol by the probiotics strains is related to the de-conjugation of the bile acids by the bile salt hydrolase enzymes (BSH). This enzyme act on the conjugated bile salts converting them into deconjugated bile salts, that can be excreted by the feces. In addition to that, the process is optimized by the compensation of the bile acids wasted in the feces by the re-up taking of the cholesterol from the blood which its already the precursor of bile acids, and using it to make up the bile salts, and as a result, the reduction in blood cholesterol will take place (12).

- **Probiotics mechanisms in exerting improved mental health**

A new term given to a group of probiotics is psychobiotics, which are microorganisms that exert positive effects on mental health. Several strains have been used in the research under this title, and they belong to the species: *Lactobacillus helveticus, Bifidobacteriumlongum, Lactobacillus casei, Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus delbrueckii subsp. bulgaricus, Bifidobacteriumbreve, Bifidobacteriuminfantis, Streptococcus salivarius, Lactobacillus rhamnosus,* and *Lactobacillus gasseri*. Many strains produce different neuro-molecules related to mood stability, that physiologically affect the host. For examples, some strains belong to *Bifidobacterium and Lactobacillus* induce the generation of gamma-aminobutyric acid (GABA) which is known for its calming effect that helps in controlling anxiety, fear and stress. Moreover, some strains of *Enterococcus, Candida, Streptococcus* and *Escherichia* generate the serotonin which is a mood stabilizer that is linked with the feelings of joy and cheerfulness, while *Bacillus* species synthesize dopamine which positively affects memory. As a result, the psychobiotics are found to reduce the feelings of anxiety, depression, aggressive thoughts, and severity of autism, while improving the mood, plasma glutathione levels, and cognitive functions(12)(13).

- **The enhancement of barrier function**

Different strategies can be implemented by probiotics resulting in an increased barrier effect. Promote mucus layer: probiotics may enhance the secretion of the mucus by increasing the mucin expression in intestinal cell lines such as (MUC2). The muscin is a glycoprotein found in the mucus in the small and large intestine and Caco-2 cell lines in the large intestine. As a result of an increased mucus production. Pathogenic microorganisms like some strains of *E.coli*, would be blocked from the adherence to the epithelial wall (14) (15).

Stimulate the integrity of the barrier: stress, pro-inflammatory cytokines, infections, can cause a reduction of the resistance of the tight junctions between the epithelial cells, and many studies showed that probiotics have the ability to stop this process (16–24). A study by Barret*et al.* found that *L. acidophilus* and *S.thermophilus* can raise the trans-epithelial resistance (TER), and reduce the permeability of the epithelial cell lines of (HT-29 and Caco-2). Moreover, these probiotics promote the activation of tight junction proteins theoccluding and ZO-1 by induced levels of the phosphorylated total proteins (25).

- **Stimulation of systemic host immunity**

Not all the probiotics give the same immune-modulatory effect to the host. They yield their effects on many cell types that are involved in the immune response, such as dendritic and epithelial cells, monocytes and the macrophages, B cells, T cells, and neutral killer (NK) cells(26)

1.2.3 Lacticaseibacillus casei ATCC 393

*Lacticaseibacilluscasei*ATCC 393 is a gram-positive bacterium, that has substantial applications in food technology and industries. The lactic acid bacterium is acid sensitive, hetero-fermentative facultative, and it is rod shaped. It is isolated from different environments such as meat, raw and fermented milk, plant products, oral, reproductive and intestinal tracts of humans and animals. This strain helps to prevent the growing of pathogenic microorganisms in the body. moreover, it improves the digestion, controls and prevents the diarrhea caused by antibiotics by its ability to keep the diversity of microbes in the gut during the antibiotic treatment and that’s because of

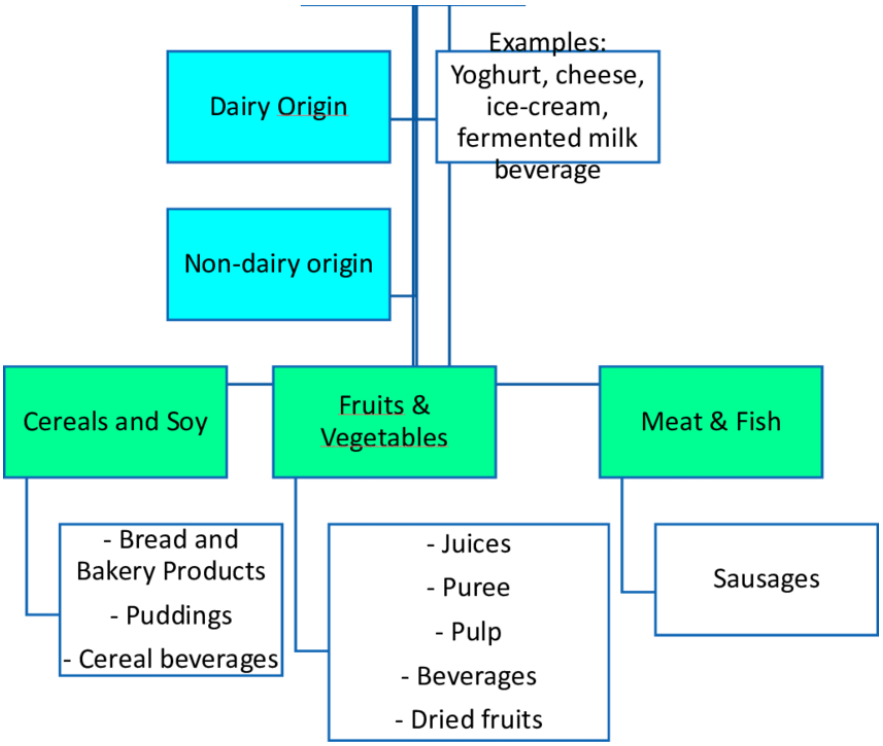
its ability of adherence to the epithelium cell wall. In addition to that it can reduce cholesterol blood levels, decreases the inflammations in the gut, reduces of lactose intolerance and the constipation symptoms. Therefore all resulting into a better immune system functioning. In addition to these health benefits, in vivo models had shown promising results upon the treatment of cancer, and the mechanism is as mentioned before, which is related to the induction of apoptosis. However, the other mechanisms are not fully understood, but other studies that investigated the ability of *L.casei*cell free supernatant to decrease the proliferation of tumor cells in colorectal cancer by reducing the metastasis process to the other organs which basically it was found to be the main reason of death (27)(28).

1.3 Problem Statement; Probiotics foods and the need to develop new ones

Traditionally, probiotic foods belong to the category of dairy products, for instance the fermented dairy products; fermented yogurts and milks. Now-a-days, manufacturers are heading towards making new plant probiotics foods, due to the increase demand of these products from the market. By this, people with specific nutritional intolerances and diseases such as lactose intolerance, or allergic to milk proteins, people with dyslipidemia, and vegans, can all include probiotics in their diet. Plant based beverages, like fruit juice could be a suitable media as carriers of probiotics. Fruit juices contains healthy nutritive ingredients like vitamins and minerals, in addition to having no allergens. Moreover, it is appealing to all age groups, by its flavor, and refreshing effect, and can be consumed in a regular basis. Despite all the mentioned above advantages, some limitations of fruit juice as vehicles of probiotics are related to the high acidity, the presence of additives such as dyes, flavors, and preservatives that are added to the juice. All of these factors can be related to the loss of cells viability. Moreover, probiotics may change the sensory characteristics of the fruit juice (1). To overcome these limitations, different authors suggested different strategies for fruit juices probiotication such as the adaptation and induction of resistance, storage under refrigeration the use of antioxidants, and attenuation techniques (29).

Figure 1.1

probiotic foods classifications(30)



1.4 Study hypothesis

1.4.1 The Attenuation strategy for probiotics

The main challenges in formulating novel probiotic foods relate to the high sensitivity of beneficial microorganisms during manufacturing and storage. Probiotics viability can be extremely affected by the exposure to the new matrix and the storage conditions. Therefore manufacturers, have spent efforts in fortifying their products with probiotics, facing all the challenges to avoid the reduction of viability to allow them to survive storage first and gastrointestinal transit later. Although the survival of probiotics is an essential factor in the formulation of probiotic foods, it is also necessary to evaluate the impact of the culture on the chemical-physical and sensory characteristics of the product. Often, consumers despite being aware of the beneficial effects associated with regular probiotic consumption, prefer the traditional product over the probiotic product because it has a better taste and smell. Recently, research has moved toward applying different strategies to limit the impact of probiotic culture on the sensory profile of the food. The attenuation strategy is considered as a system to modulate and or to control the metabolism of probiotic bacteria, and eliminate unwanted alterations in the sensory

characteristics of the food, and positively enhance the quality of the final product without affecting the viability of probiotics cells. In order to improve probiotics performance, physical or chemical treatments are suggested methods of attenuation. Although in the field of microbiology, microencapsulation has always been used as a system to protect microorganisms from harsh environments or as a transport system for probiotics, the barrier effect of the microcapsule could be exploited to attenuate microbial metabolism. In the attenuation strategy, microencapsulation can be used as a physical barrier to modulate the metabolism of the probiotics inoculated in the fruit juice, by reducing the post-acidification of the drinks during the storage time, and allows a controlled release from the core, and the diffusion processes like (nutrients and oxygen flow, and wastes products) (30) (31).

1.4.2 Microencapsulation as attenuation technology

Microencapsulation is defined as a process in which tiny particles solid and liquid or droplets are surrounded by a coating, or inserted within a homogeneous or heterogeneous matrix, in order to obtain small capsules with dimensions between millimeters and micrometers. Microcapsules, depending on the microencapsulation technique, have a spherical or irregular shape with diameter of 1-1000 μm. Previous studies describe microencapsulation in general as a way to make liquids behave as they were solids, separate the materials, decrease their toxicity, protecting the encapsulated compounds from the environmental conditions, and hide the bitter taste of some food components (32). Microencapsulation, is a way to protect probiotics from stressful environments. The microcapsule creates a microenvironment for the cell. In fact, in the microcapsule the probiotics are surrounded by a selectively permeable, thin but robust membrane. Therefore, the cells are physically separated from the external environment (food matrix). The physical separation between the matrix and the probiotics allows minimal interactions. So, the uptake of the nutrients by the probiotics is slow down and at the same time the release of probiotics metabolites, especially organic acids, is slow down too. Therefore, by appropriately changing the microencapsulation parameters and properly choosing the microencapsulation polymers, a more or less effective attenuation system can be created.

1.4.3 Microencapsulation techniques

- **Extrusion technique**

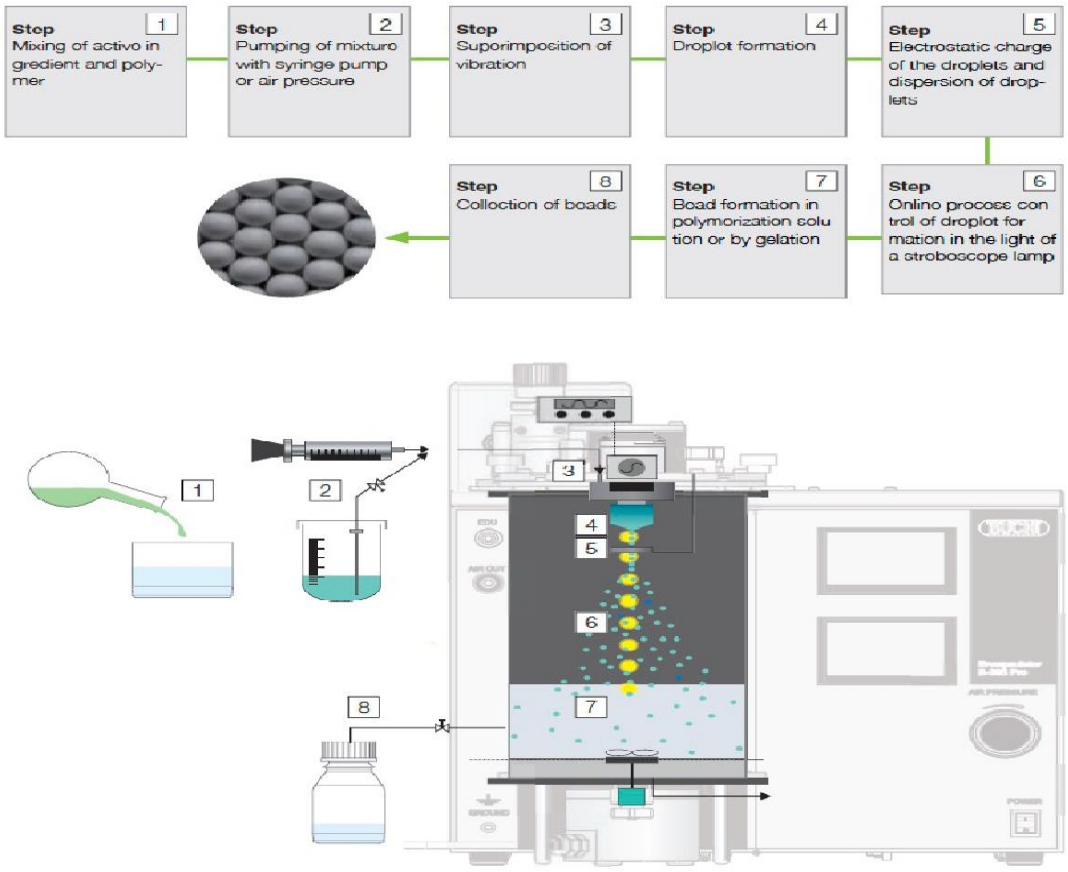
This technology is used to produce encapsulated probiotics into michrosphere. The method includes two main steps: firstly, the phase that has the probiotics is dispersed into small sized droplets, secondly these droplets become solid by gelation process or getting a membrane surrounding their surface. There are various technologies for this aim, and the selection between them depends on the production scare, maximum shear that the probiotics can tolerate, desired microsphere size, and the dispersion size as well.

- **Vibrating technology**

It's defined as the procedure which microcapsules are produced homogenously. The main principle of this technique is co-extrusion of the core and the wall by an extruder while a vibration force with specific frequency is applied to break the particles up while extrusion. As a result, the core is covered with a polymer or a material to produce the microcapsules co-axially. The extrusion of the microcapsules occurs throughout the equipment nozzle, that leads to form a laminar jet, that breaks down into different sized and shaped microcapsules due to the vibrations. In this technology, there are various ways to control the size of the microcapsules, by forming different viscosities of encapsulants mixtures, and also the concentration of the wall material, and the solidification solution used, the nozzle size, and the distance between the gelling solution and the dripping system. The nozzle which is used is made up a stainless steel cone shaped with a hole that lets the extruded polymer to pass through. The nozzle diameter can vary from 50 to 1000μm which can facilitate the production of 100 to 2000-μm microcapsules(33) (34).

Figure 1.2

Vibrating technology for microencapsulation (34).



- **Emulsion technique**

An emulsion is defined as a dispersion of one liquid into immiscible other liquid with the aid of compounds that stabilize and emulsify the two agents. If the core phase is water or aqueous, it's called water in oil (w/o) emulsion, while if the core phase is hydrophobic, then it will be called as oil in water (o/w) emulsion. The emulsions are formed by adding to core phase into the second phase (emulsifier) while being stirred vigorously. This procedure would produce very large sized capsules. The entrapment of the cells in the droplets of an emulsion, may enhance the viability of the probiotics cells, under the stressful conditions of the gastrointestinal system by facing the high acid gastric or bile salt conditions (35).

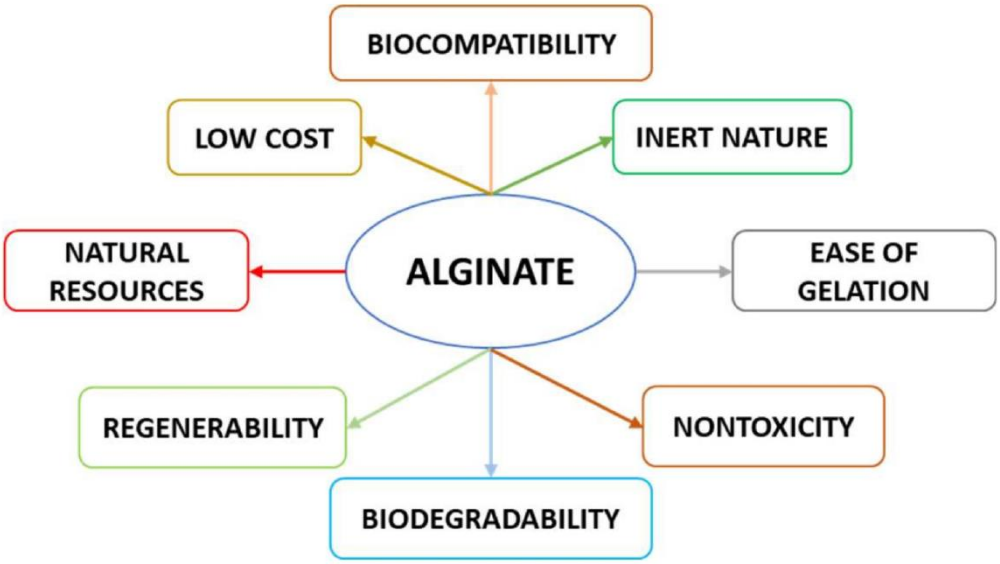
- Spray drying
- Spray cooling technique
- Coacervation
- Freeze drying technique

1.4.4 Microencapsulation polymers

Alginates: they are polysaccharides that consist of β-D-mannuronic (M)and α-L-guluronic (G) acid units and consists with sodium salts of alginic acid. There are two possible sources to obtain the alginate. The most common source is the brown algae mainly from Laminaria hyperborean, Ascophyllumnodosum, Macrocystispyifera, Laminariagigitate, Laminaria japonica, Lesonianegrescens, Sargassum sp., Eclonia maxima. Alginate can be also produced from bacteria *Pseudomonas aeruginosa*mucoid strains and *Azotobactervinelandii*. The alginates that are commercially used o come from algae sources. After extensive researches upon alginates, they had been widely used in food, in pharmaceutical, and in biomedical productions, because of their non toxic properties, the availability, easily obtained, and they are biodegradable in the human body. However, alginates have got some limitations, for example; weak barrier and mechanical stability, incompatible with heavy metals, and instable while heating. These limitations can be overcome by combining alginate with other bio-polymers, such as protein based polymers, or synthetic ones. (36)(37).

Figure 1.3

Alginates advantages (37)



Chitosan: it is a polysaccharide that is composed of D-glucosamine and N-acetyl-D-glucosamine which is obtained from crustacean shells after chitin treatment with alkaline substance such as sodium hydroxide (NaOH). According to the literature, chitosan had been one of the most popular polymer used for microencapsulation,

because it has an effect of stability upon probiotics. Moreover, chitosan plays protective role against the outer conditions of the food, therefore it protects the probiotics from harsh conditions. It also has biodegradable ability by having a significant antimicrobial and antifungal activities which were noticed in various studies. In addition to these favorable properties, chitosan has been used in microencapsulation in food industry for its physio-chemical characteristics: the amino groups gives it a positive charge which makes it commercially useful to form water-soluble cationic polymer, non toxic, and biocompatible and biodegradable for the human body (38).Table 1.2 shows other common materials used to encapsulate probiotics bacteria.

Table 1.2

common materials in encapsulating the probiotics cells (39) (40) (41) (42) (43) (44).

The polymer	Source	Mechanism of gelation	Remark
Agar	Red algae	Thermal	Resistance to degradation by most microorganisms, Low mechanical strength, High cost.
Starch	Potatoes, barely, maize, oats, etc.	Thermal	Usually blended with alginate microspheres so as to offer good protection to the bacterial cells and allow optimal diffusion of micronutrients and metabolites Decomposes under acidic conditions and presence of pancreatic enzymes in the GIT Resistant starch, used as an encapsulating polymer for probiotics, is not digested by pancreatic enzymes (amylases) in the small intestine and can be fermented in the colon.
Gellan gum	Sphingomonas elodea	Thermal/ionotropic gelation in the presence of cations	Two main types of gellan gum, acetylated (forms weak, less rigid, soft and elastic gels) and deacetylated (forms hard and brittle gels) Able to withstand the high temperature of the autoclaving process without significant loss of gel strength Acid-resistant
Gelatin	Collagen	Thermal/cross-linking using formaldehyde or glutaraldehyde/physical cross-linking using high pressure, irradiation	Useful as a thermal-reversible gelling agent for encapsulation either alone or in combination with other polymers Its amphoteric nature can be useful to form strong interaction with anionic polymer such as gellan gum when the pH is adjusted below its isoelectric point causing the net charge of gelatin to become positive
Xanthan gum	Xanthomonas campestris	Ionotropic gelation in the presence of divalent cations, commonly calcium ions	Highly resistant to enzymatic degradation, very resistant to pH variations and stable at low pHs
Milk proteins (casein, whey)	milk	Acid-induced gelation for caseins and heat induced gelation for whey proteins	Excellent gelation properties Useful for encapsulation of probiotic cells

1.5 The importance and main objectives of the study

The main objectives are to develop a microencapsulation system that can minimize/avoid sugar fermentation by probiotics and to formulate a probiotics fruit juice with similar characteristics to the traditional ones.

Chapter Two

Methods and Materials

2.1 Microorganism and growth conditions

In this research was used the probiotic *Lacticaseibacilluscasei*ATCC 393, which belongs to the culture collection of the Department of agriculture of the University of Naples Federico II. Before each assay, the microorganism was cultured under aerobic conditions into MRS broth (OXOID Ltd., Basingstoke, Hampshire, England) and incubated at 37^{o}C for 24 hours.

2.2 The attenuation of the probiotics culture

Initially, testing the attenuation of the probiotic *Lacticaseibacilluscasei*ATCC 393 had been carried out by preparing four different microcapsules from different sodium alginate concentrations. The evaluation of the maximum attenuating effect of the four concentrations of sodium alginate microcapsules was done by finding out the lowest pH level after inoculating the microcapsules into MRS broth, and measuring the pH after 6 and 24 hours of incubation at 37°C. MRS broth inoculated with*L. casei*ATCC 393 was used as a control. Results of attenuation were reported as pH decrease (ΔpH). After that, the empowering of the microcapsules was attempted by combining the sodium alginate with other polymers, or using coatings.

2.2.1 The microencapsulation process

2.2.1.1 The chemicals preparations for microencapsulation

The microencapsulation of *L. casei*ATCC 393 was done by preparing four different sodium alginate concentrations (0.8%, 1.0%, 1.2%,1.5%). Each solutions' concentration was prepared by dissolving the powder of alginic acid sodium salt from brown algae medium viscosity (*Sigma, Milan, Italy*)in deionized water then placing the solutions on heating for a complete dissolve. For the solidification of microcapsules, a solution of $CaCl_2$ (0.5 M) was used, for the breakup of the microcapsules a solution of sodium citrate (0.8 M) was used. Finally, The solutions were sterilized by autoclaving at 121 °C for 15 min.

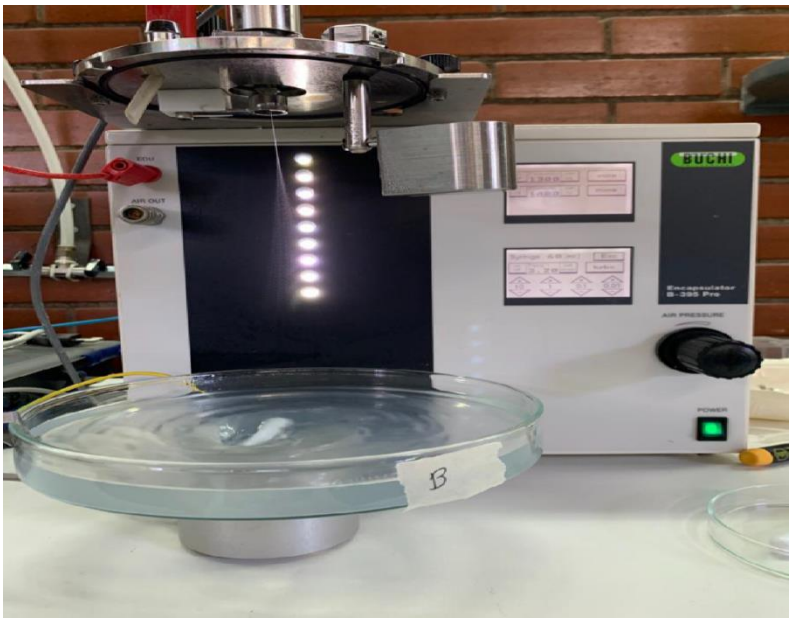
2.2.1.2 Encapsulation of *L. casei* ATCC 393

The microencapsulation of the cells of *L. casei*ATCC 393 was done by using Encapsulator B-395 Pro equipped with 120 mm nozzle and a syringe pump (BÜCHI Labortechnik, Flawil, Switzerland). The overnight culture of *L. casei*ATCC 393 was centrifuged at 8000 g for 10 min. The cell pellet was washed with Quarter-Strength Ringer solution, and finally suspended in the same volume of sodium alginate for each one of the four concentrations. The alginate-cell suspension was loaded into 60 ml syringe and then placed on the encapsulator equipment, according to the instruction of the supplier.

The parameters used in encapsulation were; vibration frequency 1100 Hz, electrode voltage 2000 mV, the jet speed was 4.30 ml/min. The alginate cell suspension was hardened in 200 ml of 0.5 M $CaCl_2$ solution for 20 min while being stirred with an agitator magnet to obtain mono-dispersed cross-linked microcapsules. The collection of the microcapsules was carried out after its sedimentation in a volumetric cylinder which contained the upper phase of $CaCl_2$ that was discarded under sterile conditions using a sterile pipette.

Figure 2.1

Encapsulator B-395 Pro equipped with 120 µm nozzle and a syringe pump



2.2.2 Improving of attenuation performances of alginate microcapsules

After finding out the alginate concentration of the best attenuating effect, there was a need to optimize the attenuation of the microcapsules, and to meliorate the morphological and functional characteristics of the microcapsules. Two strategies were applied combining the sodium alginate with another polymer, and coating with chitosan.

2.2.2.1 Mixing of sodium alginate solution with others polymers

In this procedure, whey proteins (WP) were combined with sodium alginate 0.8% solution (ALG). Three WP/ALG microcapsules were carried out, and the concentrations were illustrated below (Table $_{2.1}$).

Table 2.1

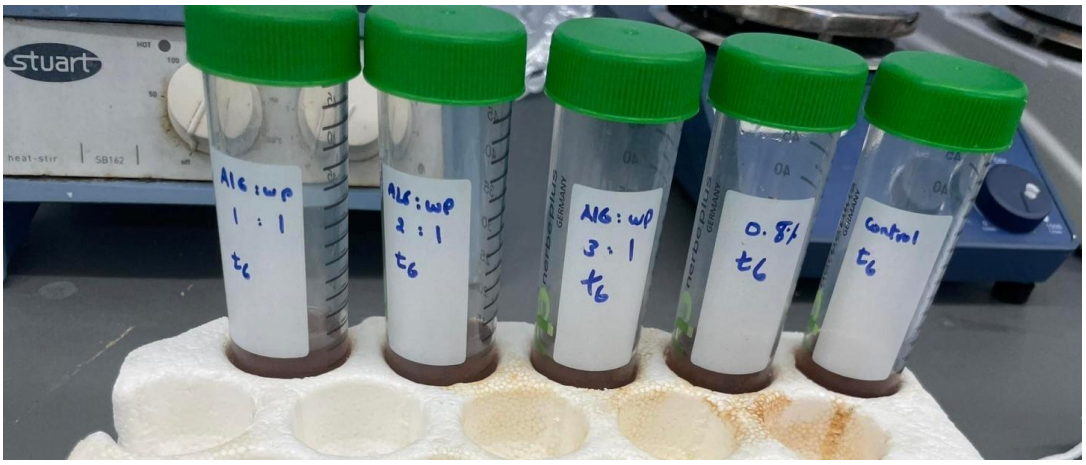
Mixing ratios of WP/ALG microcapsules

WP vs. ALG	
WP	ALG 0.8%
1	3
1	2
1	1

After this step, the microencapsulation process took place as illustrated before and the attenuation effect was evaluated with as described above.

Figure 2.2

sodium alginate 0.8% mixed with WP, in different concentrations



2.2.2.2 Chitosan coating

Chitosan: was used in order to increase the thickness of the alginate microcapsules and modify their permeability properties, the chitosan is suggested as a coating material. After choosing the best attenuation system among the four systems of sodium alginate. Alginate microcapsules were coated with chitosan (0.7 %) in ratio of 1 to 10.The coating was obtained by stirring the solution at 4500 rpm for 15 min.

- The collection of the microcapsules was carried out after its sedimentation in a volumetric cylinder.The supernatant was discarded under sterile conditions and the excess of chitosan was removed by washing step with Ringer solution.

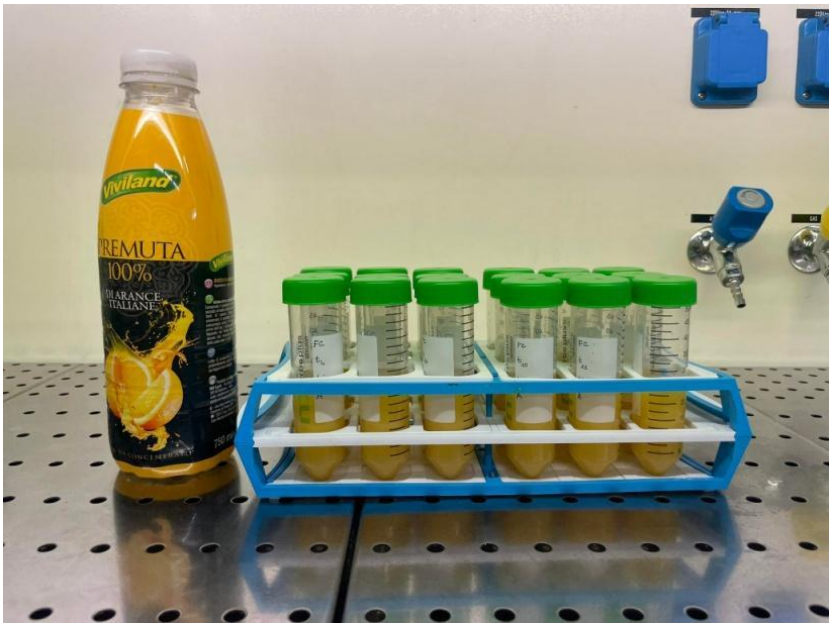
2.3 Orange juice probiotication

After testing the abilities of microcapsules to attenuate the fermentative metabolism of the probiotic *L. casei* ATCC 393 in ideal conditions (MRS broth), this system was also tested in a food matrix. A commercial orange juice was purchased from a supermarket and probioticated with two formulations:

- With a cells suspension of *L. casei*ATCC 393.
- With *L. casei* ATCC 393 as alginate microcapsules coated with chitosan.

Figure 2.3

Commercial orange juice bottle with inoculated juice samples

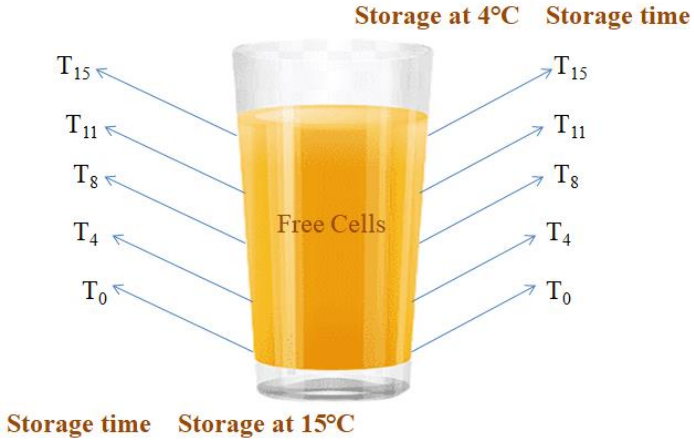


2.3.1 The probiotication of the orange juice with the *L. casei* ATCC 393 in a free form.

A fresh culture of *L. casei*ATCC 393 was centrifuged at 8000 g for 10 min. The cell pellet was then washed twice using Quarter-Strength Ringer solution, and then suspended in an equal volume of orange juice. The cell suspension was then added in the orange juice to reach a concentration of 10^7 CFU/ml. Then, the samples were stored at 4°C and 15 °C for 15 days as illustrated in figure$_{2.4}$.

Figure 2.4

Free cells probiotication



2.3.2 The probiotication of the orange juice with the microencapsulated *L. casei* ATCC 393.

A fresh culture of *L. casei*ATCC 393 was centrifuged at 8000 g for 10 min. The cell pellet was then washed using Quarter-Strength Ringer solution. The pellet was suspended into same volume of sodium alginate solution and microencapsulated as illustrate before (section 2.2.1.2). After the collection of the microcapsules, they were coated with chitosan polymer as shown before (2.2.2.2) and followed by the sedimentation and the collection of the microcapsules with sterile pipette. Finally, the microcapsules were placed into sterile falcons, and the orange juice was added to reach the original volume, to reach a concentration of 10^8CFU/ml. Then, the samples were stored at 4°C and 15 °C for 15 days as illustrated in figure$_{2.5}$.

Figure 2.5

Microencapsulated cells probiotication

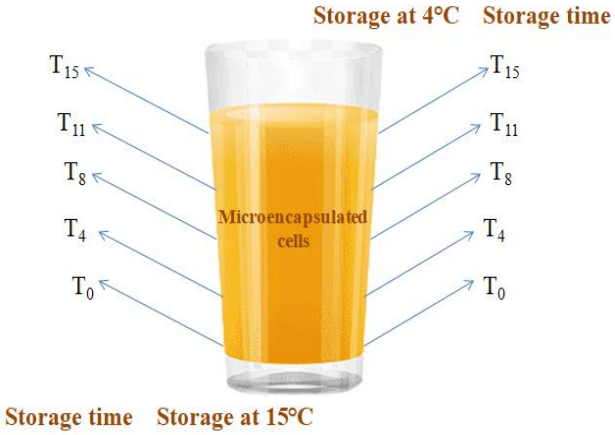
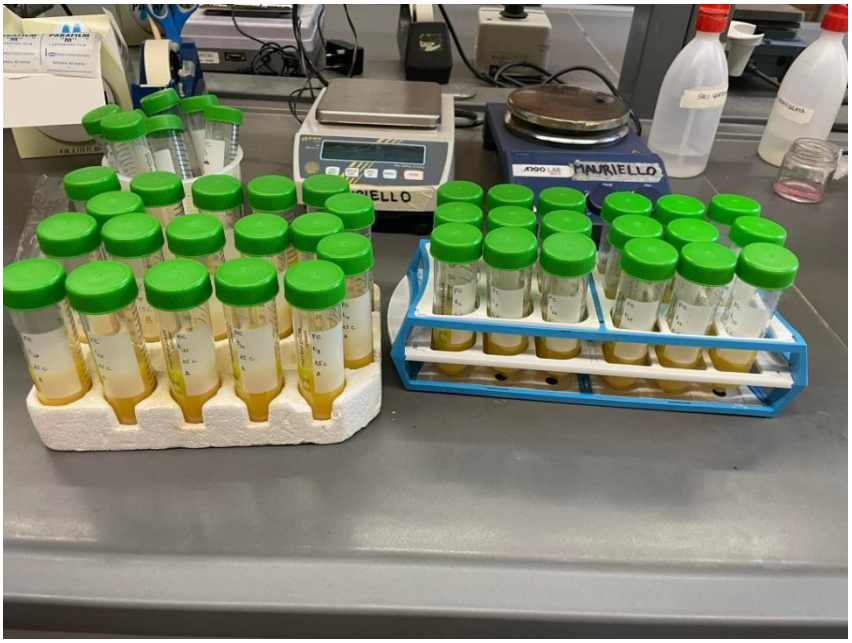


Figure 2.6

microencapsulated cells probiotication and free cells probiotication



2.4 Microbiological analysis

All the microbiological analysis were performed at t_0, t_4, t_8, t_{11} and $t_{15}$for both the juices at 4 and 15 °C. Results were reported as Log CFU/ml.

2.4.1 L. casei ATCC 393 viable count

Before and after the juice probiotication, *L. casei*ATCC 393 viable count was evaluated by making serial decimal dilutions using Quarter-Strength Ringer solution. The probiotic was cultured on MRS agar by the spread plate method plates were incubated at 37^{o}C for 48-72 hours. The same procedure was used to monitoring the probiotic viability during juice storage.Before to count the cells inside the microcapsules, these were break using 0.2 M sodium citrate solution. So, for the serial decimal dilutions was used a first tube of sodium citrate to break the microcapsules. The rest of the dilutions were done in Ringer solution.

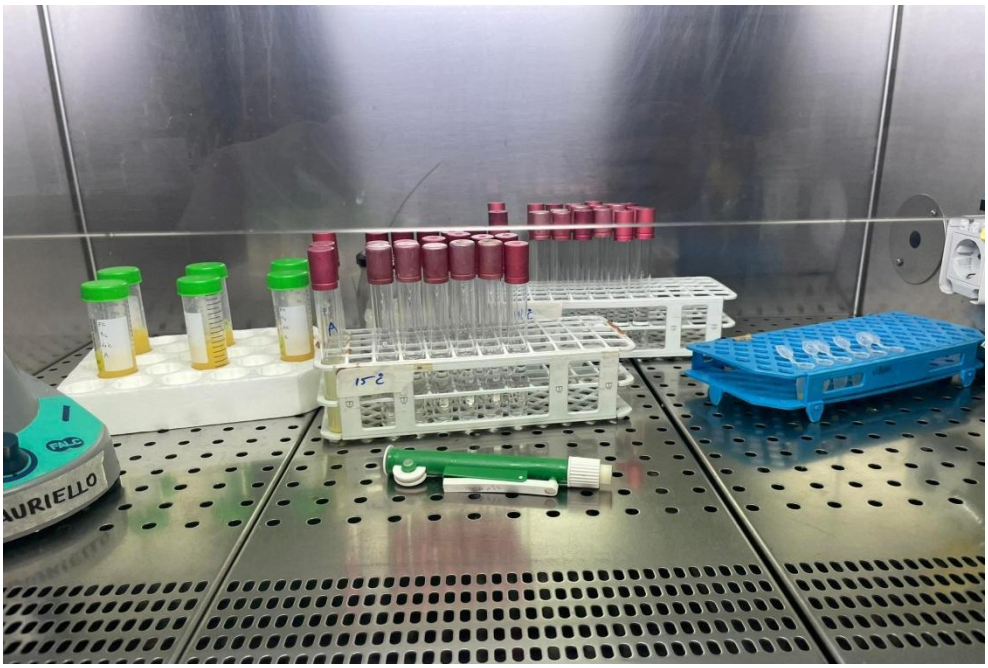
2.4.2 Fruit juice microbiological quality

During the juices storage others microbiological analysis were performed to assess the safety of the products. Total mesophilic aerobic bacteria (TMAB) were counted on Plate Count Agar (PCA) by spread plate method and the plates were incubated at 37 ° C for 48-72 h. PCA was also used to count the Total Psychrophiles Aerobic Bacteria (TPAB).

The plates were incubated at 5-7 °C for 7-10 days. Finally, Malt Extract Agar (MEA) was used for molds and yeasts (28 °C for 3-5 days).

Figure 2.7

Serial dilutions



2.5 Physio-chemical analysis

All the physico-chemical analysis were performed at t_0, t_4, t_8, t_{11} and t_{15} for both the juices at 4 and 15 °C.

2.5.1 Titratable acidity (TA) test

25 ml of the sample was placed in a beaker with 3-4 dropsof phenolphthalein 1%, and 75 ml of deionized water was added while stirring by a magnet agitator. The solution was titrated drop by drop with 0.1 M NaOH, until the phenolphthalein was viridescent. Results were reported as citric acid g/100 ml of sample through the formula (1):

The formula was used to calculate titratable acidity.

(1)$TA = \frac{V \times N \times 0{,}064}{m}$

Where:

V is the volume of NaOH in ml

N is the normality of NaOH solution

m is the weight of the sample in grams

0,064 is the equivalent weight of citric acid

2.5.2 Ascorbic acid (AA) test

First of all, to quantify the content of ascorbic acid in juice sample, an ascorbic acid standard solution was prepared by dissolving 0.25 g of ascorbic acid in 250 ml of deionized water. The titration was done by adding 100 ml of deionized water to 25 ml of the standard solution. The, 1 ml of starch solution (2 %) was added as indicator. Lugol solution was used as titrant and added drop by drop until a dark color appeared. The test was carried out by placing 25 ml of the sample in a beaker and then the same procedure was followed. Results were reported as g of ascorbic acid.

The g of ascorbic acid were calculated through the formula (2)

(2) g AA = $\frac{0.25}{5.4} x\ V$

V is the volume of lugol solution in ml

2.5.3 The Color test

A 15 ml of the samples were poured in a sterile Petri dish. The colorimeter was used to measure the color coordinates L*, a* and b*.

Results were expressed as color variation (ΔE) by formula (3):

(3) $\Delta E = \sqrt{(L_t - L_0)^2 + (a_t - a_0)^2 + (b_t - b_0)^2}$

Where L_t, a_t, b_t are the colorimetric coordinates of the juice at different analysis times, and L_0, a_0, b_0 are the colorimetric coordinates at time zero.

the samples were categorized as:

Verydifferent$\Delta E > 3$

Different$\Delta E = 3$

Slightlydifferent$\Delta E < 3$

2.5.4 Total soluble solids test (tss)

A refractometer was used for the measurement of Total Soluble Solids (TSS) by adding few drops of the samples into the slide of the refractometer. Results were reported as °Brix.

2.5.5 pH measurement

The samples were placed into falcons and used for pH measurement by a pH meter.

2.5.6 Statistical analysis

All treatments were carried out in three independent batches. In each case, an untreated sample was used as a control. one-way ANOVA and t-test analysis were performed to determine significant differences between averages using statistical analysis system (IBM-SPSS). Significance will be declared at $P < 0.05$.

Chapter Three

Results and Discussions

3.1 Alginate microcapsules

3.2 Attenuation induced by alginate microcapsules

The attenuation effect of *L. casei*Atcc 393 metabolism was mainly evaluated by measuring the pH decrease of MRS broth inoculated the probiotic in free and microencapsulated forms. Results of the attenuation induced by ALG microcapsules with different ALG concentration (0.8 %, 1.0%, 1.2 %, 1.5 %) are reported in $Table_{3.1}$. The minimum decrease of pH was 0.28 and 1.81 for A_0.8 sample after 6 and 24 h of incubation, respectively. However, no significant differences ($p > 0.05$) were found between microencapsulated and non-microencapsulated probiotic. In another words, the microencapsulation with alginate and the alginate concentration has no effects on the reduction of pH of MRS broth compared with the controlsample LC(*L. casei*Atcc 393). Probably, this phenomenon is correlated to the microcapsule structure. The microcapsule mechanical and structural properties depend on the type of alginate used and on the gelatinization process. When alginate reacted with Ca^{2+} ions the gelatinization immediately occurred. This lead to the formation of heterogeneous gels with porous surface (45). The numbers and the dimension of these porous made the out layer define the permeability of the structure. We can suppose that the microcapsules of alginate, independently of its concentration, had a lax structure that allowed nutrients to enter and consequently acidify the culture medium.

Therefore, it was decided to improve the performances of the microencapsulation as attenuationsystem by combining the alginate with another polymer and with a coating. Moreover, to buildalso the cheapness system, sodium alginate 0.8% was chosen for the microencapsulation process.

Decrease in MRS broth pH following inoculation of the probiotic strain *Lacticaseibacilluscasei* ATCC 393 (control) and microencapsulated samples. LC: *L. casei* ATCC 393, control; A_0.8: *L. casei* ATCC 393 microencapsulated in 0.8 % sodium alginate; A_1.0: *L. casei* ATCC 393 microencapsulated in 1.0 % sodium alginate; A_1.2: *L. casei* ATCC 393 microencapsulated in 1.2 % sodium alginate;

A_1.5: *L. casei* ATCC 393 microencapsulated in 1.5 % sodium alginate. Results are reported as mean values of three replicates ± standard deviation. Same letters in the same column indicate that the differences are not significant ($p < 0.05$, One-way ANOVA).

Table 3.1

Attenuation induced by sodium alginate microcapsules at different concentrations.

Sample	ΔpH t_6	ΔpH t_{24}
LC	0.46± 0.15[a]	2.02± 0.08[a]
A_0.8	0.28± 0.08[a]	1.81 ±0.18[a]
A_1.0	0.31± 0.13[a]	1.87 ± 0.12[a]
A_1.2	0.35 ± 0.12[a]	1.88 ± 0.16[a]
A_1.5	0.33± 0.14[a]	1.95± 0.17[a]

3.3 Alginate microcapsules characterizations

Alginate microcapsules were observed at light microscope at 400 x magnification. Comparing these images, it is clear that the increase of alginate concentration induces morphological changes. 0.8 % alginate microcapsules have a pedunculated shape with ill-defined margins. Instead, the 1.5 % alginate microcapsules (Figure$_{19}$) have a perfectly spherical shape and well-defined margins.

The capsules of *L. casei*ATCC 393 were produced with an approximate diameter 240 μm with a continuous surface of cell membrane was resulted from a nozzles' diameter size (120 μm) that was used for microencapsulation using vibrating technology (Encapsulator B-395). The literature approved that the size of capsules is larger than the diameter of used nozzle.

3.4 The effect of combining WP with sodium alginate 0.8%

The main target of combining other polymers such as WP with sodium alginate was to enhance the attenuation effect of the microcapsules, since microencapsulation with sodium alginate 0.8% had no effects on the reduction of pH of MRS broth compared with the control sample LC (*L. casei*ATCC 393). WP was tested in three concentrations combining sodium alginate 0.8% to produce better attenuating microcapsules. The ratios of WP:AlG were illustrated in Table $_{2.2}$ (2.2.2.1). The results in table $_{3.2}$ show that

the minimum decrease of the pH of MRS broth was 0.32 and 1.69 for A_0.8; probiotics microencapsulated with 0.8% alginate sample after 6 and 24 h, respectively. However, no significant difference ($p>0.05$) were found in the reduction of pH of MRS broth between A_0.8 and the three different concentrations of WP combined with 0.8% sodium alginate (M_1, M_2, M_3) microcapsules and with control sample LC (*L. casei*ATCC 393). Therefore, combining the WP with ALG was not used as an enhancement strategy for the attenuation effect of the microcapsules.

Decrease in MRS broth pH following inoculation of the probiotic strain *Lacticaseibacilluscasei* ATCC 393 (control) and microencapsulated samples. LC: *L. casei* ATCC 393, control; A_0.8: *L. casei* ATCC 393 microencapsulated in 0.8 % sodium alginate; M_1: ALG/WP 3:1 microcapsules; M_2: ALG/WP 2:1 microcapsules; M_3: ALG/WP 1:1 microcapsules; Results are reported as mean values of three replicates ± standard deviation. Same letters in the same column indicate that the differences are not significant ($p < 0.05$, One-way ANOVA).

Table 3.2

Attenuation induced by sodium alginate (ALG) microcapsules in combination with serum protein (WP).

Sample	ΔpH t_6	ΔpH t_{24}
LC	0.45± 0.04[a]	1.85± 0.33[a]
A_0.8	0.32± 0.03[a]	1.69±0.27[a]
M_1	0.42± 0.04[a]	1.80± 0.32[a]
M_2	0.42± 0.08[a]	1.81± 0.34[a]
M_3	0.44 ± 0.06[a]	1.83± 0.31[a]

3.5 WP-Alginate microcapsules characterizations

WP-Alginate microcapsules were observed under light microscope at 400 x magnification. Comparing the A_0.8 microcapsules with the ones with the addition of whey protein, it can be noticed that the M_1, M_2 and M_3 microcapsules had a pedunculatedand irregular shape. Moreover, the ALG/WP microcapsules would appear to have a rough surface. The images are reported.

The capsules of *L. casei*ATCC 393 with WP and 0.8% alginate were produced with an approximate diameter 240μm with a continuous surface of cell membrane was resulted from a nozzles' diameter size (120 μm) that was used for microencapsulation using vibrating technology (Encapsulator B-395).

3.6 The effect of chitosan coating of sodium alginate 0.8% microcapsules

The coating with chitosan was used as a strategy to enhance the attenuation effect of the sodium alginate 0.8% microcapsules. The minimum decrease of pH of the MRS broth was 1.48 for the chitosan coated microcapsules (CMC) after 24h of incubation at 37 °C. Moreover there was a statistically significant difference ($p < 0.05$) in pH reduction between the CMC the A_0.8 sample, and control. In another words, the coated microcapsules with chitosan had significantly the least reduction effect in pH of MRS broth compared with control sample LC when incubated for 24 h. While there was no significant difference ($p > 0.05$) found between the reduction of pH of MRS broth between sample control LC and the sample A_0.8, after 6 and 24 h of incubation, and also for CMC after 6h of incubation. Probably, the chitosan coating improves the structural characteristics of the alginate microcapsules by going to cover the pores of the microcapsule. This leads to effective slowing of nutrient uptake and subsequent release of metabolites that result in pH change. Therefore, the CMC system represents a valuable attenuation system. Accordingly with these results,Accordingly, CMC were used in the probiotication of the orange juice.

Decrease in MRS broth pH following inoculation of the probiotic strain *Lacticaseibacilluscasei* ATCC 393 (control) and microencapsulated samples. LC: *L. casei* ATCC 393, control; A_0.8: *L. casei* ATCC 393 microencapsulated in 0.8 % sodium alginate; CMC: microcapsules with chitosan coating. Different letters in the same column indicate that the differences between the samples are significant ($p < 0.05$, One-way ANOVA).

Table 3.3

Attenuation induced by sodium alginate (ALG) microcapsules coated with chitosan (CHI).

sample	ΔpH t_6	ΔpH t_{24}
LC	0.43 ± 0.08^{a}	2.13 ± 0.13^{a}
A_0.8	0.35 ± 0.09^{a}	1.93 ± 0.26^{a}
CMC	0.38 ± 0.11^{a}	1.48 ± 0.10^{b}

3.7 Chitosan coated microcapsules characterizations

Chitosan coated microcapsules were observed at light microscope at 400 x magnification. Comparing these images, it is clear that the coating with chitosan produced regular perfect spherical shaped and well-defined margins of the microcapsules, in contrast when using 0.8% alginate microcapsules without coating.

3.8 Orange juice physiochemical characteristics

3.8.1 pH values during the storage

The pH values of the samples of orange juice inoculated with free cells *Lacticaseibacilluscasei* ATCC 393 (LC_OJ) and with the microencapsulated *Lacticaseibacilluscasei*ATCC 393 (MC_OJ) at T_0 were, 3.30 ± 0.01 and 3.28 ± 0.04 respectively, with no significant ($P \geq 0.05$) differences. Figure A.13 (in Appendix A) shows the results of pH for the probiotic juices at 4 °C.

For both probiotic juices, at 4 °C storage the pH values remained approximately constant with no significant differences ($p \geq 0.05$). However, at t_{11} a significant difference was found between the two samples. In particular, LC_OJ reach the lowest pH value of 3.23± 0.04 while for the MC_OJ sample the pH was 3.29± 0.03. These results are correlated with the low metabolic activity of the probiotic under refrigerated conditions. Therefore, attenuation by microencapsulation is not necessary under refrigerated storage.

Results of pH values for the 15 °C storage were reported in Figure A.14 (in Appendix A). Contrary to expectation, the pH of the LC_OJ sample stored at 15 °C also remained constant throughout the storage period. Only at t_{15} there was a slight increase in pH (3.37 ± 0.01). Probably, the probiotic metabolized some juice constituents that lead to the release of smaller molecules that contribute to the increase of pH.The MC_OJ presented a constant pH value as expected. However, also for the 15 °C storage it is no necessary to develop an attenuation system.

At the end, there is no significant ($P \geq 0.05$) difference in pH values between the two juices from the start of the storage time until the final time.Therefore, the attenuating effect of the microencapsulation for the *L. casei* ATCC 393 in the orange juice stored at the two temperatures, is not different when the juice is probioticated with the free cells.

Probablythese results depend on the type of probiotic used and of the type of juice. Environments with low pH could represent a stress for probiotic cells inducing a slowdown in metabolic activity. Moreover, orange juice has a low pH. Therefore, the probiotic is unable to metabolize the sugars present and further lower the pH.

3.8.2 Titratable acidity of the juice

The titratable acidity (TA) was determined by titrating with 0.1 N NaOH, and expressed as g of citric acid/100 ml of products. The citric acid values of LC_OJ and MC_OJ at T_0 were 1.07 ± 0.03 and 1.02 ± 0.04 respectively. Figure A.15 (in Appendix A) shows the trend of titratable acidity during refrigerated storage.

Although the pH of samples LC_OJ and MC_OJ at 4 °C remained almost constant, the titratable acidity, on the other hand, varied over time. In particular, the MC_OJ sample exhibited significantly lower titratable acidity than the LC_OJ juice. Although, at t_4, t_8 and t_{11} there was a significant reduction in titratable acidity for the juice fortified with the probiotic in the form of microcapsules, at t_{15} statistical analysis revealed no significant differences. In this case, the cells in free form exhibit greater metabolic activity than microencapsulated cells. The results obtained for probiotic juices stored at 4 °C were similar to those obtained for probiotic juices stored at 15 °C Figure A.16 (in Appendix A). As expected, at 15 °C the LC_OJ juice shows an increase in titratable acidity that is correlated with higher metabolic activity favored by the higher storage temperatures. Microencapsulation appears, therefore, necessary to attenuate probiotic metabolism thereby reducing sugar metabolization and minimizing changes in TA. However, at 15 days of storage no significant differences were found between the two samples. Probably, this phenomenon could depends on the breaking of some microcapsules and the release of *L. casei* ATCC 393 into the juice.

3.8.3 Ascorbic acid of the juice

Ascorbic acid contents of samples were determined according to the titration method. The ascorbic acid value of LC_OJ andMC_OJ at T_0 were 0.15 ± 0.02 and0.13 ± 0.00 respectively, with no significant ($P \geq 0.05$) differences.

At storage temperature 4 °C Figure$_{27}$, there is a reduction in ascorbic acid quantities, or described as a depletion of vitamin C content of the juice, where this matter attributed to the loss during the storage and sample preparations (47). In addition to these results, no significant differences were found between the two juices in ascorbic acid amounts, in another words, microencapsulation of probiotics had no effects on ascorbic acid comparing with LC_OJ. Although there are no significant differences between the samples, the reduction of ascorbic acid in MC_OJ juice shows a linear trend. Whereas, for LC_OJ juice, a drastic reduction can be seen at t_{11}, which continues until the end of storage at T_{15}.

At storage temperature 15 °C figure$_{28}$,no significant differences ($P > 0.05$) were found between the two juices in ascorbic acid amounts. However, at T_{11}, there was a significant ($P > 0.05$) reduction in ascorbic acid amount by LC_OJ, which means that microencapsulation significantly ($P > 0.05$) slowed down the ascorbic acid reduction. In addition to that, in the LC_OJ sample *L. casei* ATCC 393 leads to a more rapid consumption of ascorbic acid along the storage. However, at the end of the storage time, microencapsulation of probiotics had no effects on ascorbic acid content compared with LC_OJ, and the reduction of ascorbic acid continues to be linear for both juices.

3.8.4 Color

Table$_{3.4}$ shows the results of color variation (ΔE) during the storage of probiotic juices for borh temperature. All the samples, for all the time of monitoring presented a $\Delta E < 3$. This means that regardless of the form of addition and storage temperature, probiotication does not result in any color changes.

LC-OJ 4 °C: orange juice added with probiotic in free form stored at 4 °C; MC-OJ 4 °C: orange juice added with probiotic in free form stored at 4 °C;LC-OJ 15 °C: orange juice added with probiotic in free form stored at 15 °C;MC-OJ 15 °C: orange juice added with probiotic in free form stored at 15 °C. Data are reported as mean values (n = 3).

Table 3.4

Changes in colour of all juice samples at different storage time.

Sample	Days	ΔE		
		< 3	= 3	>3
		Slightly different	Different	Very different
LC-OJ 4 °C	4	+	-	-
	8	+	-	-
	11	+	-	-
	15	+	-	-
MC-OJ 4 °C	4	+	-	-
	8	+	-	-
	11	+	-	-
	15	+	-	-
LC-OJ 15 °C	4	+	-	-
	8	+	-	-
	11	+	-	-
	15	+	-	-
MC-OJ 15 °C	4	+	-	-
	8	+	-	-
	11	+	-	-
	15	+	-	-

3.9 Microbial analysis

3.9.1 L. casei ATCC 393 viable counts

The viable count of *L. casei* ATCC 393 in the LC_OJ and MC_OJ samples was shown in figure$_{29}$. At T_0 were 8.12 ± 0.17 and 8.29 ± 0.10 Log UFC/ml respectively, with no significant ($P \geq 0.05$) differences.

During the refrigerated storage, no significant differences ($p > 0.05$) were found between LC_OJ and MC_OJ samples. The same results were found for the juices stored at 15 °C.Moreover, for both juices there has been a slight decline in the viability. This result goes in parallel with the result obtained from the pH values during the storage, because of the direct relation between the viable count and the pH changes during the storage.Thus, the combination of this probiotic strain with orange juice is also an excellent combination for the vitality parameter. So, microencapsulation turns out not to be necessary for maintaining the viability of the probiotic. Instead, which retains, both at 4 and 15 °C unchanged viability.

3.9.2 Moulds and yeasts

Further analysis was conducted to ascertain the microbiological safety of orange juices. Table$_{3.5}$, and table$_{3.6}$ show the increase of the growth of moulds and yeasts in MC_OJ stored at 4 °C. And in MC_OJ stored at 15 °Crespectively. In contrast, in LC_OJ juice stored at both 4 and 15 °C, the microbial load for mold and yeast always remained zero. The presence of mold and yeast in the juices fortified with the probiotic in the form of microcapsules is related to contamination at the process stage. In fact, the microencapsulation was carried out under non-sterile conditions. Although careful cleaning of the machinery and work surfaces was carried out, this was not sufficient to prevent environmental contamination. on the other hand, LC_OJ juices were produced under sterile conditions by always working under a laminar flow hood. This supports the hypothesis of environmental contamination.

LC_OJ 4 °C: orange juice with *L. casei* ATCC 393 in free form stored at 4 °C; MC_OJ 4 °C: orange juice with *L. casei* ATCC 393 as ALG microcapsules coated with chitosan stored at 4 °C; LC_OJ 15 °C: orange juice with *L. casei* ATCC 393 in free form stored at 15 °C; MC_OJ 15 °C: orange juice with *L. casei* ATCC 393 as ALG microcapsules coated with chitosan stored at 15 °C. Results are reported as mean values ± standard deviation (n =3).Different letters in the same raw means that the differences between the samples are significant.

Table 3.5

Moulds counts during probiotic fruit juice storage.

Days	Moulds (Log UFC/ml)			
	LC_OJ 4 °C	MC_OJ 4 °C	LC_OJ 15 °C	MC_OJ 15 °C
0	0^{a}	0.36 ± 0.32^{a}	0^{a}	0^{a}
4	0^{a}	0.56 ± 0.47^{b}	0^{a}	0^{a}
8	0^{a}	0.73 ± 0.67^{b}	0^{a}	0^{a}
11	0^{a}	0.97 ± 0.83^{b}	0^{a}	0^{a}
15	0^{a}	1.49 ± 1.29^{b}	0^{a}	0^{a}

LC_OJ 4 °C: orange juice with *L. casei* ATCC 393 in free form stored at 4 °C; MC_OJ 4 °C: orange juice with *L. casei* ATCC 393 as ALG microcapsules coated with chitosan stored at 4 °C; LC_OJ 15 °C: orange juice with *L. casei* ATCC 393 in free form stored at 15 °C;MC_OJ 15 °C: orange juice with *L. casei* ATCC 393 as ALG microcapsules coated with chitosan stored at 15 °C. Results are reported as mean values ± standard

deviation (n =3).Different letters in the same raw means that the differences between the samples are significant.

Table 3.6

Yeasts counts during probiotic fruit juice storage.

Days	Yeasts (Log UFC/ml)			
	LC_OJ 4 °C	MC_OJ 4 °C	LC_OJ 15 °C	MC_OJ 15 °C
0	0^{a}	0^{a}	0^{a}	0^{a}
4	0^{a}	0^{a}	0^{a}	1.28 ± 0.98^{b}
8	0^{a}	0^{a}	0^{a}	1.34 ± 1.00^{b}
11	0^{a}	0^{a}	0^{a}	1.49 ± 1.11^{b}
15	0^{a}	0^{a}	0^{a}	2.87 ± 1.64^{b}

3.9.3 Total Psychotropic Aerobic Bacteria (TPAB) counts

Table$_{3.7}$ shows a significant growth in TPAB counts for MC_OJ 4 °C and MC_OJ 15 °C, during the storage. The increase in the growth of TPAB in the MC_OJ and not in the samples with free cells probiotics is due to the same reasonaided for the growth of moulds and yeasts which it is the microencapsulating conditions.

LC_OJ 4 °C: orange juice with *L. casei* ATCC 393 in free form stored at 4 °C; MC_OJ 4 °C: orange juice with *L. casei* ATCC 393 as ALG microcapsules coated with chitosan stored at 4 °C; LC_OJ 15 °C: orange juice with *L. casei* ATCC 393 in free form stored at 15 °C; MC_OJ 15 °C: orange juice with *L. casei* ATCC 393 as ALG microcapsules coated with chitosan stored at 15 °C. Results are reported as mean values ± standard deviation (n =3).Different letters in the same raw means that the differences between the samples are significant.

Table 3.7

Total Psychotropic Aerobic Bacteria (TPAB) counts during probiotic fruit juice storage.

Days	TPAB (Log UFC/ml)			
	LC_OJ 4 °C	MC_OJ 4 °C	LC_OJ 15 °C	MC_OJ 15 °C
0	0^{a}	0^{a}	0^{a}	0
4	0^{a}	2.76 ± 0.93^{b}	0^{a}	3.43 ± 0.17^{c}
8	0^{a}	3.15 ± 0.61^{b}	0^{a}	3.50 ± 0.13^{c}
11	0^{a}	3.18 ± 0.63^{b}	0^{a}	3.55 ± 0.11^{c}
15	0^{a}	3.23 ± 0.56^{b}	0^{a}	3.71 ± 0.23^{c}

3.9.4 Total Mesophilic Aerobic Bacteria (TMAB) counts

Figure A.21 and A.22 (in Appendix A) show the results for the TMAB count. Results for TMAB counts during both storages remained constant for LC_OJ. It is important to mention that the TAMB and the viable counts of *L. casei* ATCC 393 for LC_OJ samples were similar to each other during the storage. Thus, it is possible to say that actually the TMAB is represented by the probiotic. However, for the MC_OJ at the TMAB counts was 5.19 ± 0.10 Log UFC/ml. An aliquot with intact microcapsules was used to count TMAB in MC_OJ juice. Analyses conducted on commercial juice (data not shown) did not detect the presence of TMAB. Given that intact microcapsules can also give rise to colonies when grown on agarized culture medium, it is possible to state that the results obtained are due to the presence of the microcapsules in the sample. In addition, for the MC_OJ sample stored at 4 °C at t4, there is an increase in the charge probably due to the breakdown of the microcapsules. The same phenomenon but with greater intensity was recorded for the MC_OJ sample stored at 15 °C. These results together with those obtained for LC_OJ juices support the hypothesis that the results obtained in the total mesophilic count are due to the presence of the probiotic rather than the development of othermicroorganisms.

Chapter Four

Conclusions

The addition of probiotic microorganisms to plant-based food matrices has limitations due to the matrix-probiotic interaction and the consequent modification of the matrix itself limiting consumer acceptance. This study was based on the hypothesis that through the application of an attenuation system on probiotic cultures, matrix modification can be avoided/slowed down. Therefore, by exploiting the barrier properties of microcapsules, microencapsulation was proposed as an attenuation system for the probiotic *Lacticaseibacilluscasei* ATCC 393. Preliminarily, several microcapsules were developed. First by varying the concentration of sodium alginate; then by combining sodium alginate with whey proteins; and finally, by coating the alginate microcapsules with a chitosan solution. The results obtained show that the concentration of sodium alginate has no influence on the fermentative metabolism of the probiotic. In addition, the combination with whey protein did not lead to improved performance of the microcapsules as an attenuation system. Instead, sodium alginate microcapsules coated with chitosan (CMC) were shown to be a good attenuation system. After studying microencapsulation as an attenuation strategy under ideal conditions (MRS broth), we moved on to a food application. Orange juice was chosen as a vegetable beverage to be probioticate. The probiotic was added to the orange juice in free form (LC_OJ) and in the form of microcapsules (MC_OJ). During 15 days of storage at 4 and 15 °C, physical-chemical and microbiological parameters were monitored. The results obtained showed no significant differences ($P < 0.05$) between LC_OJ and MC_OJ juices. In fact, pH, TA, AA content, TSS and color remained unchanged during storage at both 4 and 15 °C. Furthermore, for both storage conditions, in LC_OJ juice the probiotic presented constant viability values. Therefore, the viable count of *L. casei* ATCC 393 remained constant even without a protection system such as microcapsules. Although in preliminary tests CMC microcapsules were necessary to minimize the effects of *L. casei* ATCC 393 metabolic activity on the pH of MRS broth, the opposite result was recorded in the case of orange juice. The results obtained demonstrate the potential of microencapsulation as an attenuation technology. However, the use of microcapsules for the attenuation of probiotic cells for the probiotication of an orange juice did not lead to the expected results. In addition, the results of probiotication of orange juice have shown that there is no need for an attenuation

system. In fact, the LC_OJ presented a high count of *L. casei* ATCC 393 (8 Log UFC/ml) while maintaining constant physical-chemical characteristics not just during the refrigerated storage but also at 15°C. In conclusion, considering the viability of the probiotic, the physicochemical parameters, and the non-need for refrigeration, orange juice was found to be a suitable matrix for the probiotication with *L. casei*ATCC 393.

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Appendices

Appendix B

Figures of Study

Figure A.1

Different plates and medias for cells count

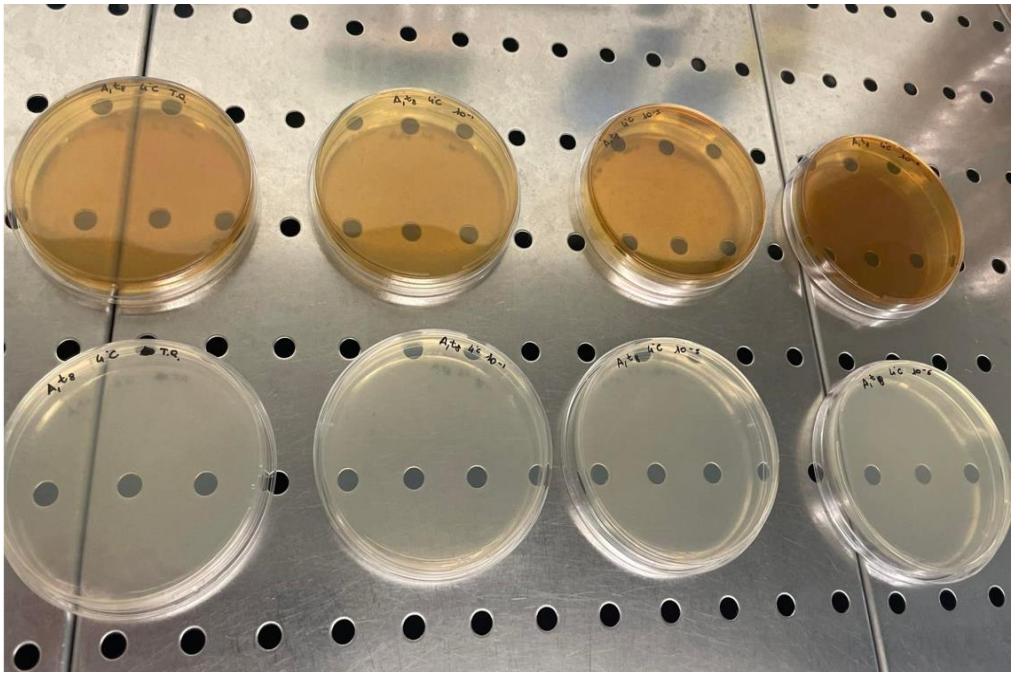


Figure A.2

sample during titratable acidity test

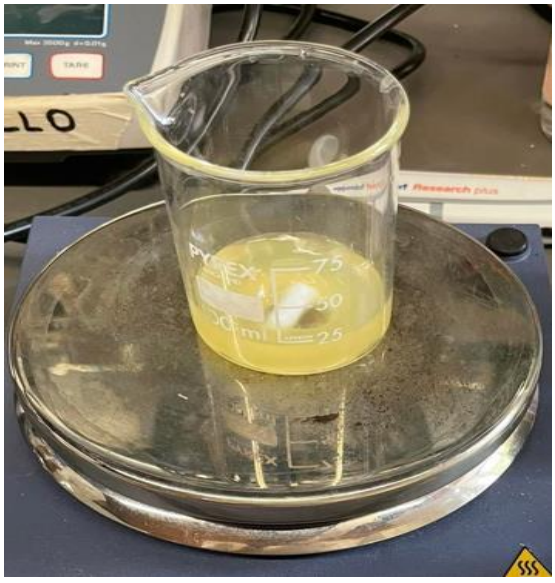


Figure A.3

sample during ascorbic acid test

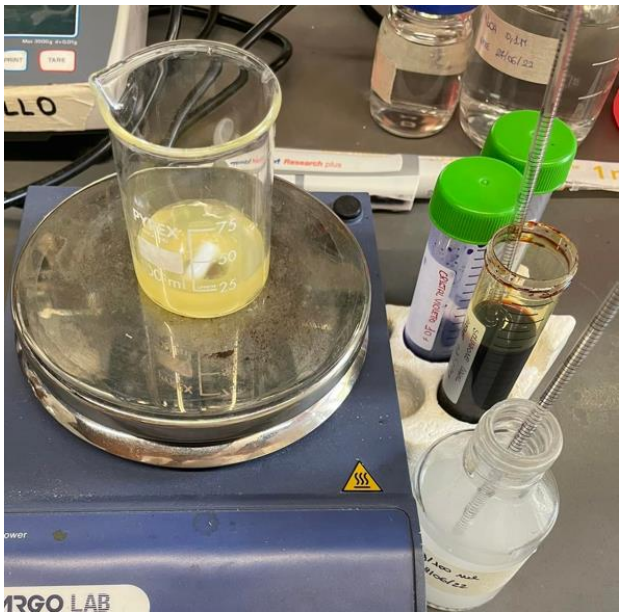


Figure A.4
the pH meter

Figure A.5

0.8% ALG microcapsules at light microscope 400 X magnification.

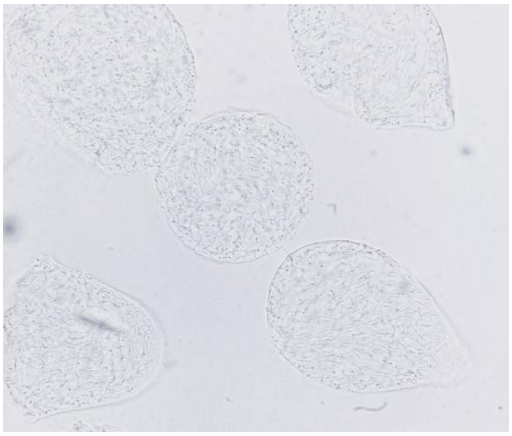


Figure A.6

1.0% ALG microcapsules at light microscope 400 X magnification.

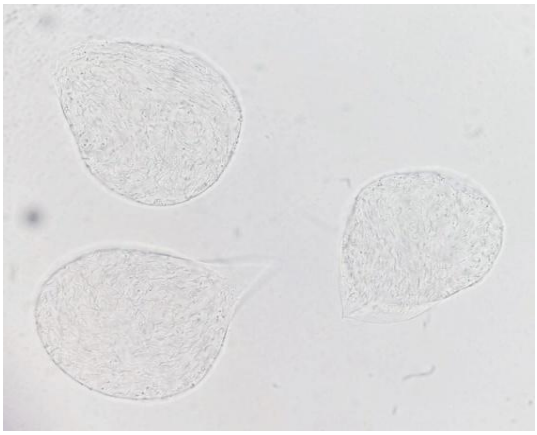


Figure A.7

1.2% ALG microcapsules at light microscope 400 X magnification.

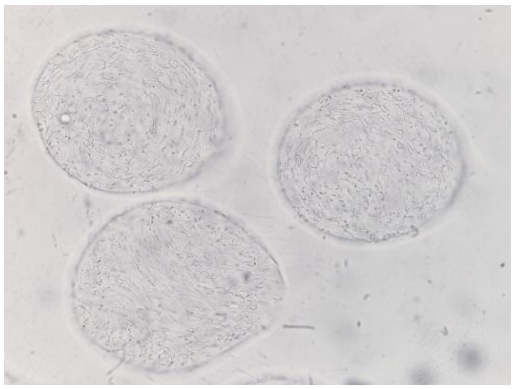


Figure A.8

1.5% ALG microcapsules at light microscope 400 X magnification.

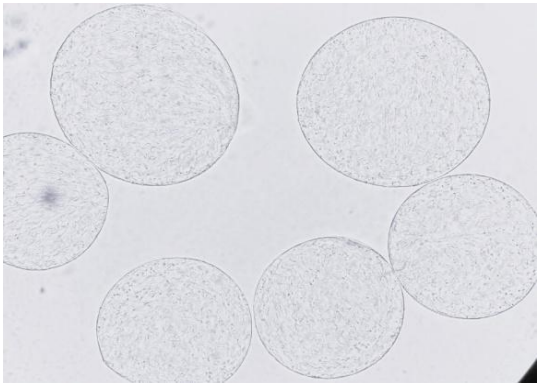


Figure A.9

M1: ALG/WP 3:1 microcapsules microcapsules at light microscope 400 X magnification.

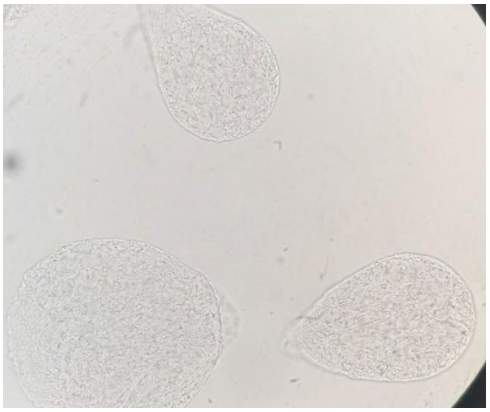


Figure A.10

M2: ALG/WP 2:1 microcapsules microcapsules at light microscope 400 X magnification.

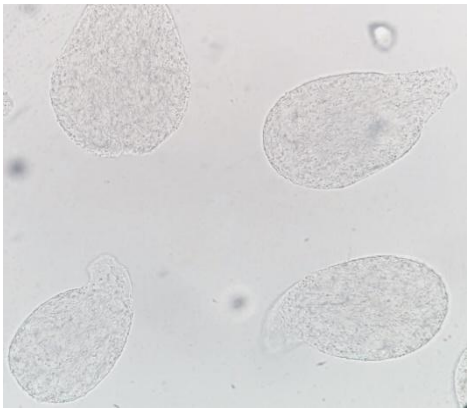


Figure A.11

M3: ALG/WP 1:1 microcapsules microcapsules at light microscope 400 X magnification

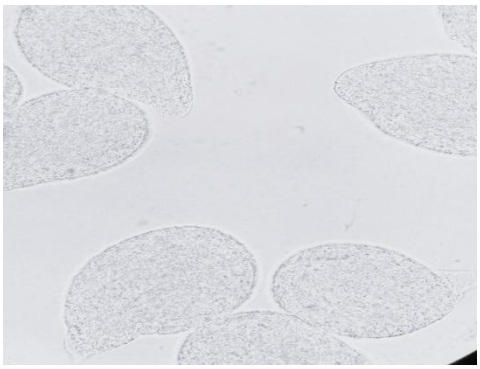


Figure A.12

ALG 0.8% coated with chitosan microcapsules, with regular well defined-margins under light microscope 400 X magnification

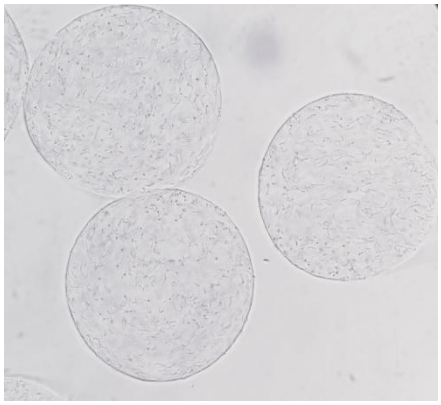


Figure A.13

pH of orange juice containing free L. casei ATCC 393 (LC_OJ) and microencapsulated L. casei ATCC 393 (MC_OJ) during 15 days of storage at 4°C Values are reported as the means of three experiments ± standard deviation. For each storage time different letter indicates that the differences between the samples are significant (P ≥ 0.05).

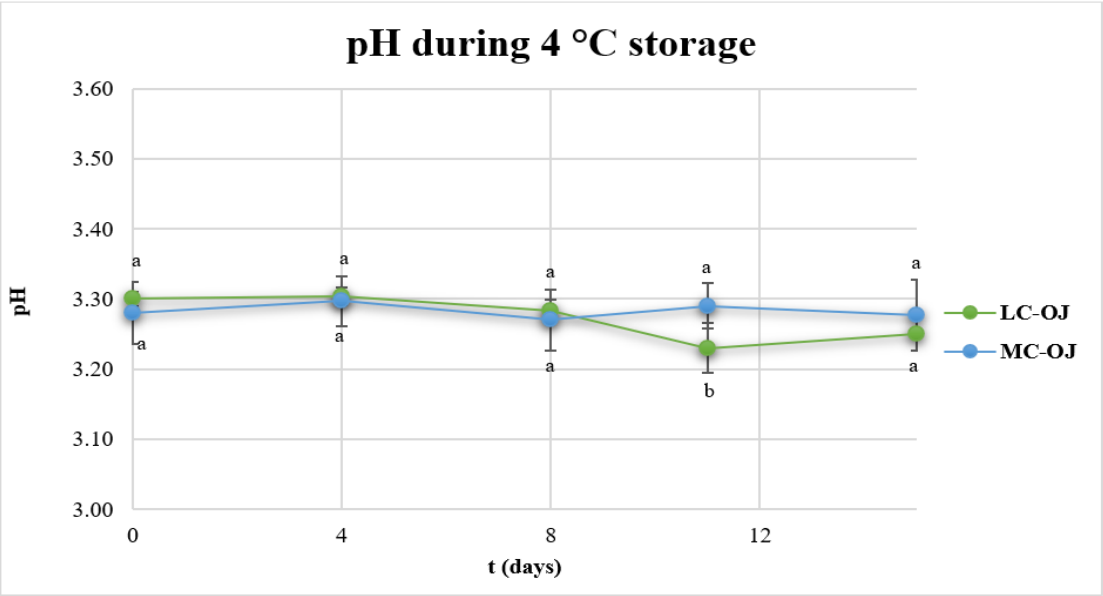


Figure A.14

pH of orange juice containing free (LC) and microencapsulated (MC) L. casei ATCC 393 during 15 days of storage at 15°C. Values are reported as the means of three experiments ± standard deviation. For each storage time different letter indicates that the differences between the samples are significant (P ≥ 0.05).

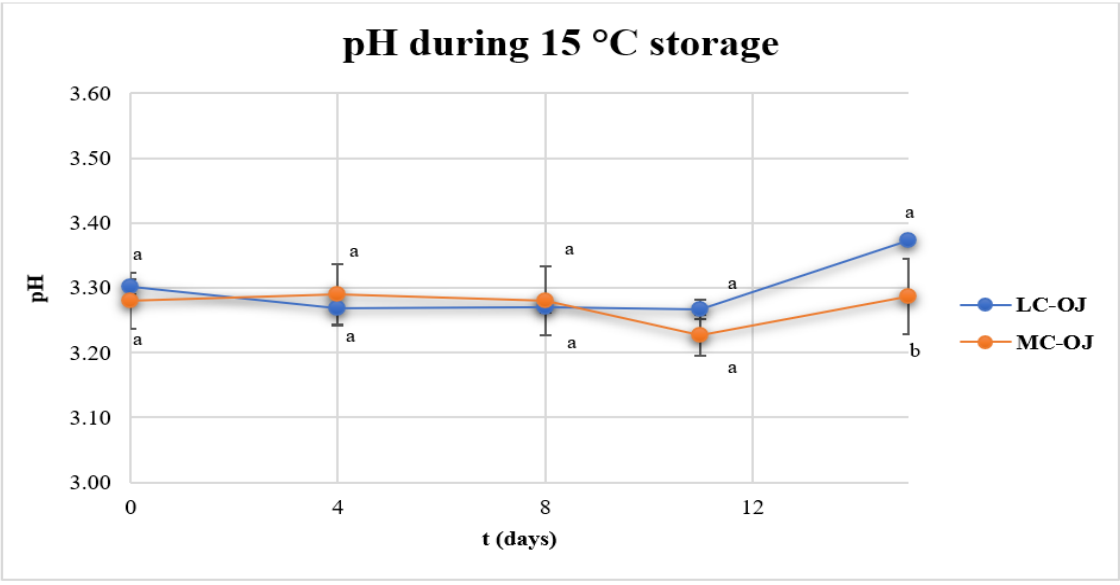


Figure A.15

titratable acidity of orange juice containing free (LC) and microencapsulated (MC) L. casei ATCC 393 during 15 days of storage at 4°C. Values are reported as the means of three experiments ± standard deviation. For each storage time different letter indicates that the differences between the samples are significant (P ≥ 0.05).

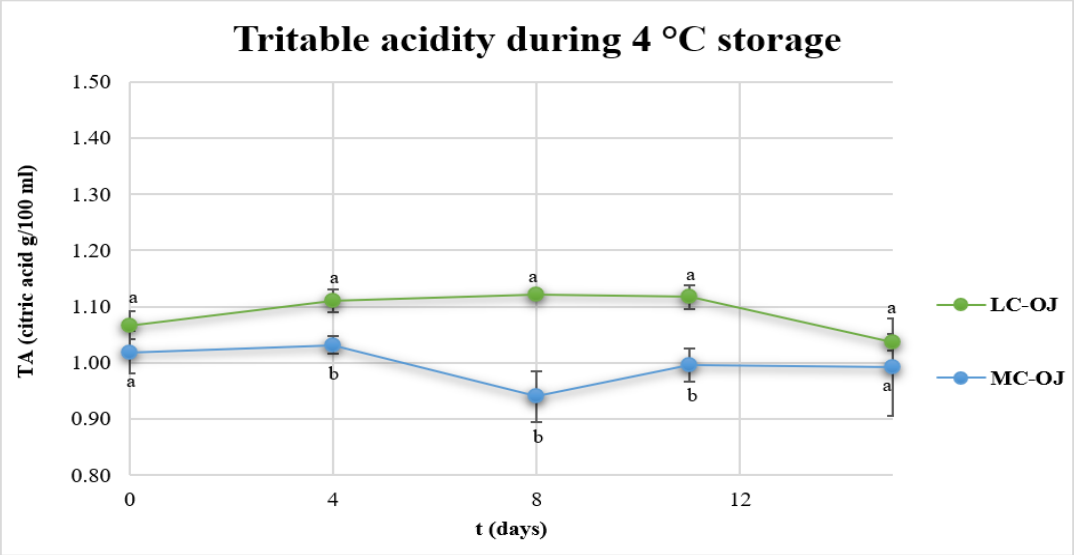


Figure A.16

titratable acidity of orange juice containing free (LC) and microencapsulated (MC) L. casei ATCC 393 during 15 days of storage at 15°C. Values are reported as the means of three experiments ± standard deviation. For each storage time different letter indicates that the differences between the samples are significant (P ≥ 0.05).

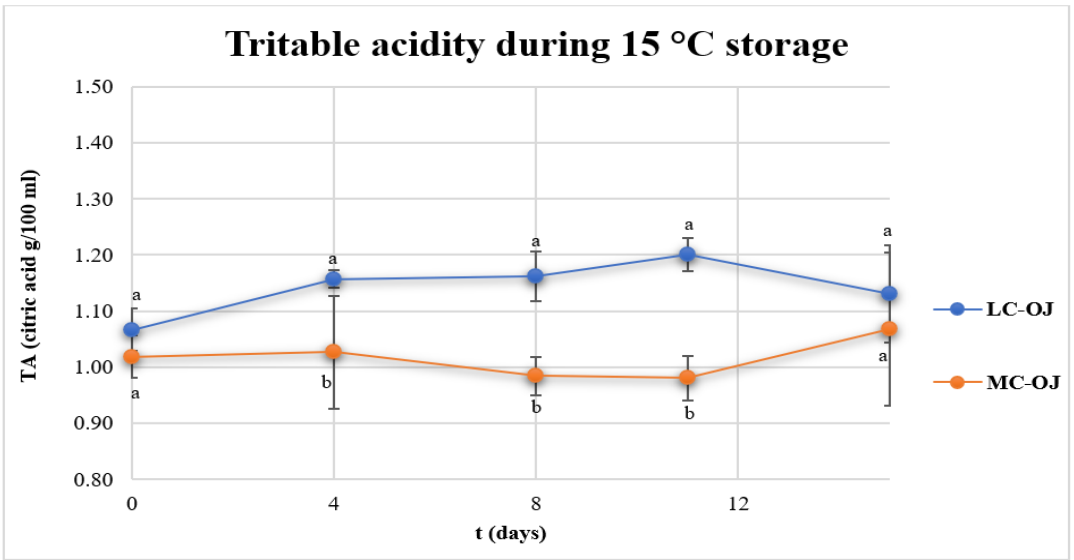


Figure A.17

Ascorbic acid in orange juice containing free (LC) and microencapsulated (MC) L. casei ATCC 393 during 15 days of storage at 4°C. Values are reported as the means of three experiments ± standard deviation. For each storage time different letter indicates that the differences between the samples are significant (P > 0.05).

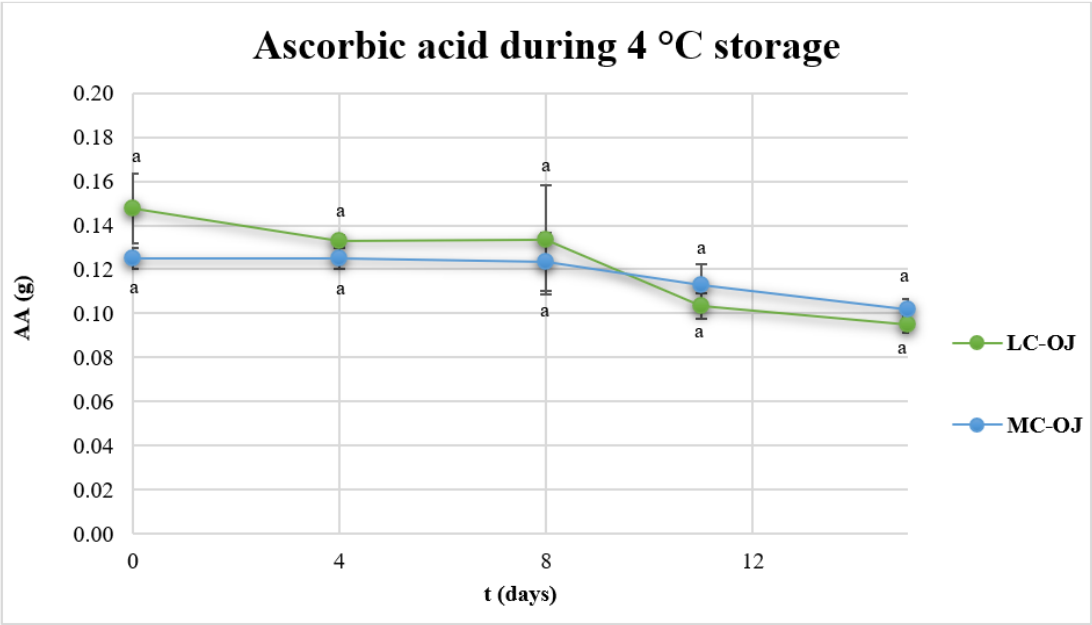


Figure A.18

Ascorbic acid in orange juice containing free (LC) and microencapsulated (MC) L. casei ATCC 393 during 15 days of storage at 15°C. Values are reported as the means of three experiments ± standard deviation. For each storage time different letter indicates that the differences between the samples are significant (P > 0.05)

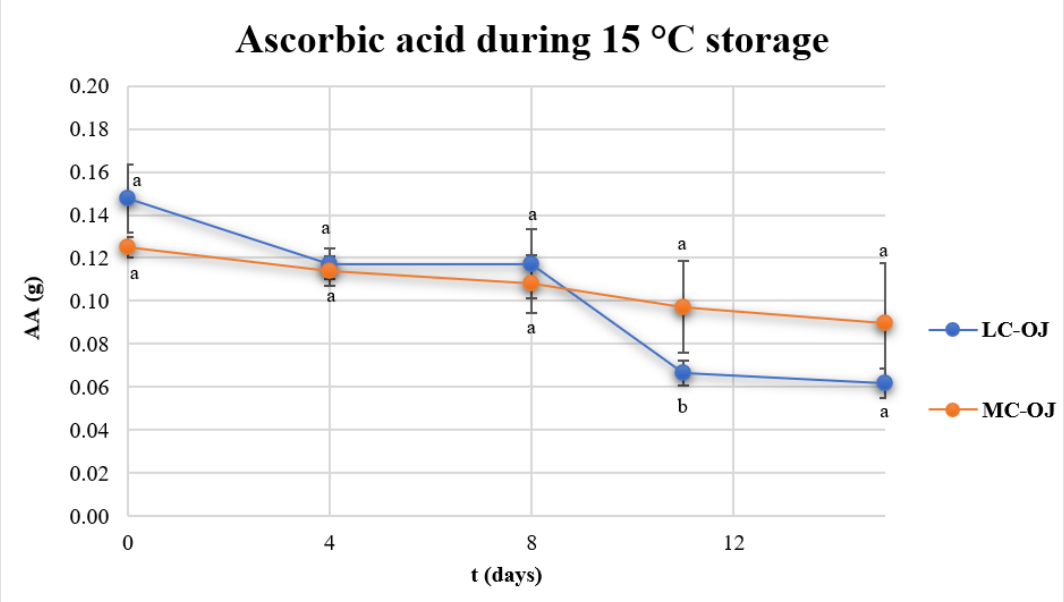


Figure A.19

L. casei viable count during storage in orange juice containing free (LC_OJ) and microencapsulated (MC_OJ) L. casei ATCC 393 during 15 days of storage at 4°C. Values are reported as the means of three experiments ± standard deviation. For each storage time different letter indicates that the differences between the samples are significant (P < 0.05).

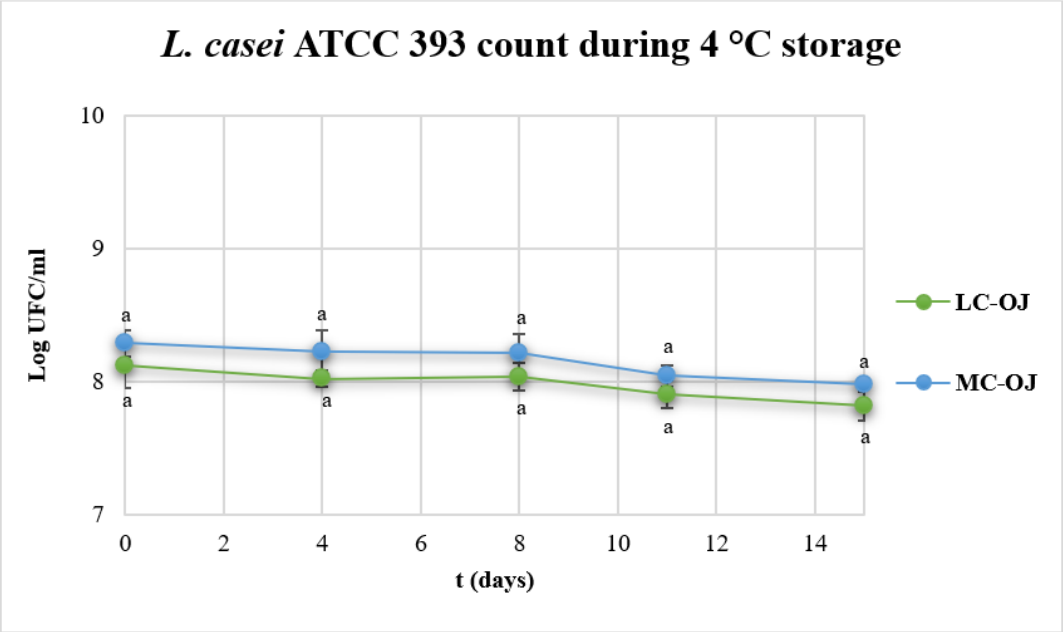


Figure A.20

L. casei viable count during storage in orange juice containing free (LC) and microencapsulated (MC) L. casei ATCC 393 during 15 days of storage at 15°C. Values are reported as the means of three experiments ± standard deviation. For each storage time different letter indicates that the differences between the samples are significant (P < 0.05).

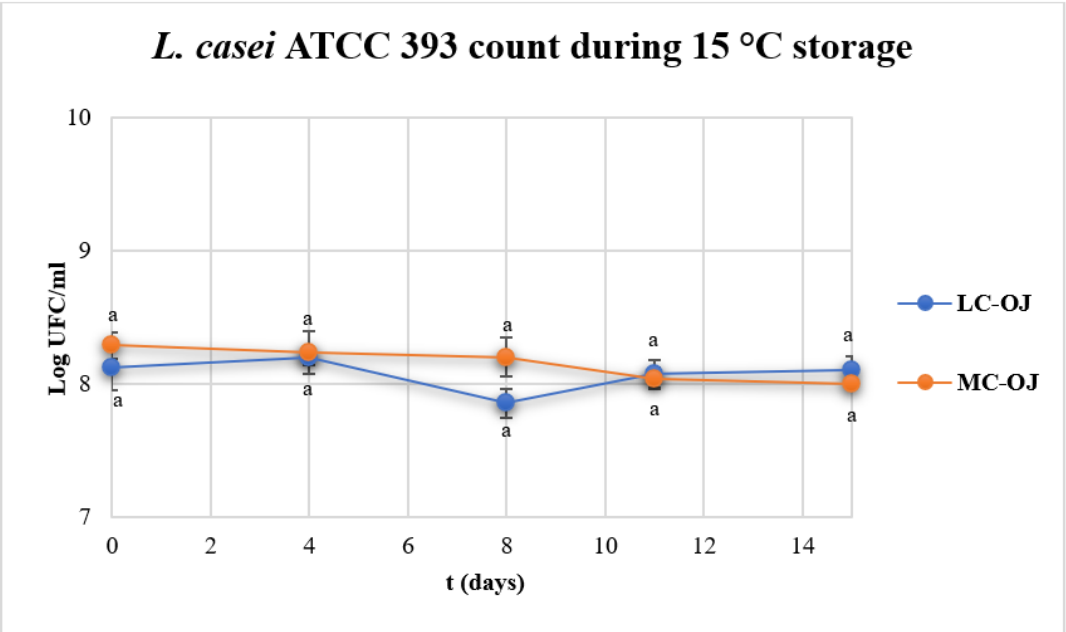


Figure A.21

L. casei viable count during storage in orange juice containing free (LC) and microencapsulated (MC) L. casei ATCC 393 during 15 days of storage at 4°C. Values are reported as the means of three experiments ± standard deviation. For each storage time different letter indicates that the differences between the samples are significant (P < 0.05).

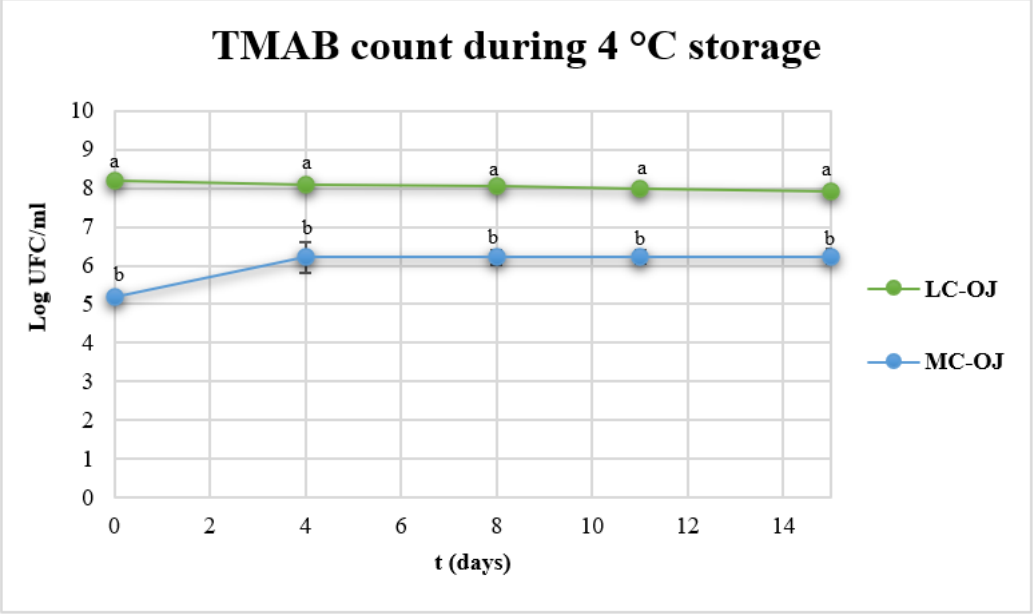
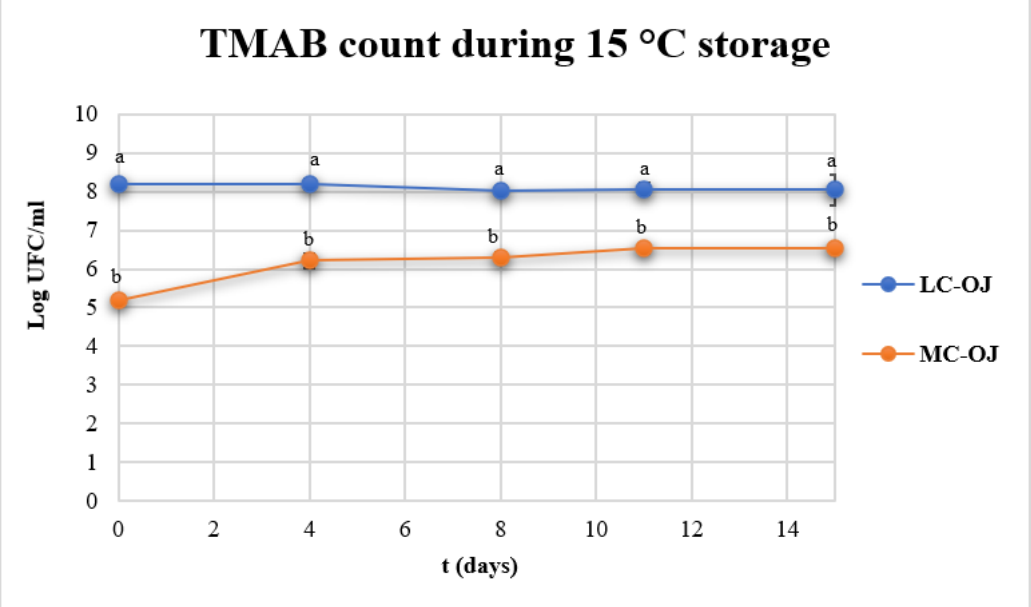


Figure A.22

L. casei viable count during storage in orange juice containing free (LC) and microencapsulated (MC) L. casei ATCC 393 during 15 days of storage at 15°C. Values are reported as the means of three experiments ± standard deviation. For each storage time different letter indicates that the differences between the samples are significant (P < 0.05).



تخفيف النشاط الاستقلابي للبكتيريا النافعة عن طريق استخدام الكبسولة الدقيقة باستراتيجية الاستحلاب

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الملخص

خلفية الدراسة: تعتبر عملية اضعاف او التخفيف من نشاط بكتيريا البروبيوتيك عن طريق استعمال استراتيجية الكبسولة الدقيقة وسيلة لعزل البكتيريا فيزيائيا عن بيئة الغذاء الموجودة فيه، وذلك عن طريق اعادة تعديل النشاط الايضي لها.

الهدف الرئيسي من هذه الدراسة هو دراسة تأثير اضعاف او التخفيف باستعمال الكبسولة الدقيقة على عملية التمثيل الغذائي للسلالة البكتيريا **(*Lacticaseibacilluscasei* ATCC 393)**. بعد ذلك، تمت عملية تطبيق التخفيف هذه باستعمال نظام التخفيف الذي تم العمل عليه لادراج بكتيريا البروبيوتيك في عصير البرتقال، لاختبار فعالية الكبسلة الدقيقة لتجنب التغيرات الفيزيائية والكيميائية للعصير أثناء تخزينه. **منهجية الدراسة;** في هذه التجربة، تم عمل نوعين من هذا العصير بحيث: تمت إضافة**LC_OJ** من البروبيوتيك بشكل حر وإضافة**MC_OJ** من البروبيوتيك على شكل كبسولات دقيقة تتكون من ألجينات الصوديوم بنسبة 0.8٪ مغلفة بالكيتوزان، بالإضافة لذلك تم اجراء اختبارات ميكروبيولوجية وفيزيوكيميائية للعصيرين أثناء التخزين لمدة 15 يومًا على 4 درجات مئوية و 15 درجة مئوية.

النتائج: لم يكن هناك أي تغيير على درجة الحموضة عند استعمال الكبسلة الدقيقة للبروبيوتيك عند تبريد العصير. ومع ذلك كانت لدى عينة عصير البرتقال**MC_OJ** حموضة معايرة أقل **(TA)** من**LC_OJ** لكل من درجتي حرارة التخزين، مما يعني أن الكبسلة الدقيقة خفضت **TA**. في هذه الدراسة، لم تظهر عملية ادخال البروبيوتيك الى العصير أي تأثير على تغير اللون عند عصير البرتقال بكلتا الحالتان. وينطبق الشيء نفسه على محتوى حمض الأسكوربيك، وذلك بسبب فقدان محتوى فيتامين سي أثناء تحضير العينات وتخزينها. أظهرت النتائج النهائية لـ**LC_OJ** و**MC_OJ** أن الكبسلة الدقيقة لـ ***L. casei*ATCC 393** لم يكن لها تأثير على عملية الاضعاف. بالإضافة الى ان كل من **LC_OJ** و**MC_OJ** احتوى على أعدادًا عالية بكتيريا البروبيوتيك.

النتائج: ان عندما يضاف بروبيوتيك ***L. casei* ATCC 393** إلى عصير البرتقال لا يوجد داع للتوهين او الاضعاف. علاوة على ذلك، يمثل عصير البرتقال بيئة مناسبة للمحافظة على بكتيريا البروبيوتيك بحيث يحفظ الخصائص الكيميائية والفيزيائية الثابتة للعصير مع مرور الوقت وفي نفس الوقت يضمن بقاء بكتيريا البروبيوتيك.

كلمات مفتاحية: الاضعاف (التوهين)، *L.casei* ATCC 393، عصير البرتقال، اعداد البكتيريا، درجة الحموضة.