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Effect of encapsulation in sodium alginate, k-carrageenan and chitosan on the viability of some Lactic Acid Bacteria during storage in juice and gastrointestinal conditions.

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II- MATERIALS AND METHODS

III- RESULTS AND DISCUSSION

CAP	Cellulose Acetate Phthalate
CDs	Cyclodextrins
CFU	Colony forming unit
E	Encapsulated
F	Free
GIC	Gastro Intestinal Conditions
LAB	Lactic acid bacteria
ME	Microencapsulation
WP	Whey Protein

Introduction

In recent years, consumers awareness towards the relationship between food and health has led to an explosion of interest in functional foods which are fortified with ingredients capable of producing health benefits. In this context, the addition of living probiotic microorganisms to food is a prominent way to create functional foods**.** LAB are the most important probiotic that have an important role in food, agricultural and clinical applications and beneficial effect in the ecosystem of the human intestinal tract **(Heidebach *et al*., 2012; Harel and Tang, 2014; Perricone *et al*., 2015; Bintsis, 2018).**

The use of probiotic bacteria is not limited to dairy product but is extended to other forms of functional foods or beverages **(Dianawati *et al*., 2015)**. Recently, beverages based on fruits juices have been reported as a novel and appropriate medium for probiotic, for their content of essential nutrients and do not contain starter cultures that compete for nutrients with probiotics as they can combine the appearance of healthy and fresh foods (**Perricone *et al*., 2015; Hruyia *et al*., 2018)**.

Probiotics should be metabolically stable and active in the product, survive passage through the stomach and reach the intestine in large amounts **(Al-Furaih *et al*., 2016).** However, the loss of living probiotic cell numbers during processing, storage, and gastro intestinal transit caused by various stress factors is an important issue and has to be avoided **(Heidebach *et al*., 2012).**

Microencapsulation of probiotics has been investigated for improving their viability by creating a physical barrier against harsh environmental conditions during manufacturing process of food and those encountered during gastric juice passage to reduce cell injury or cell lose, in a way that results in appropriate cell release in the gut **(Krasaekoopt *et al*., 2004; Al-Furaih *et al*., 2016; Jantarathin *et al*., 2017).**

In this work, we aim to study the effect of encapsulating method using different polymers on the viability of some lactic acid bacteria during storage under different temperatures and in pineapple juice, and to test if they can resist the simulated gastrointestinal conditions.

Chapter I.Litterature review

I.1. Lactic acid bacteria

I.1.1. Definition

Lactic acid bacteria (LAB) constitutes a group of Gram positive bacteria characterized by certain morphological, metabolic, and physiological characteristics **(Siezen et *al*., 2002)**. They are chemotrophic, aero-anaerobe facultative or microaerophiles, rod or cocci shaped, non-spore forming and non-motile bacteria. Also, they showed positive tests of indole, methyl-red and nitrate reduction, and negative tests of catalase and oxidase production and citrate-utilization **(Dhamale *et al*., 2015; Jagadeesh, 2015)**. They have complex exigences such as growth factors, amino acids, peptides, puric and pyrimidic bases, vitamins B and fatty acids **(Bekhouche, 2006).**

LAB have the capacity to produce lactic acid only for the homofermentative type using the Embden-Meyerhof-Parnas pathway, some times more than lactic acid, other compounds like acetic acid, ethanol and carbonic gas are produced for the heterofermentative type via hexose monophosphate or pentose pathway **(Bekhouche, 2006; Dhamale *et al*., 2015).**

I.1.2. Habitat

LAB are widespread microorganisms which can be found in any environment rich mainly in carbohydrates, such as plants, fermented foods (fermented meat and fish …), dairy products, beverages, sewage, mucosal surfaces of humans and in cavities (mouth, genital, intestinal and respiratory tract) of human and animals **(Florou-Paneri, 2013; Konig *et al*., 2017)**.

I.1.3. Classification

The classification of LAB into different genera is largely based on morphology, mode of glucose fermentation, growth at different temperatures, configuration of the lactic acid produced, ability to grow at high salt concentrations, and acid or alkaline tolerance as well as fatty acid composition and cell wall composition, lactic acid isomers from glucose, behavior against oxygen, arginine hydrolysis, bile tolerance, type of hemolysis, production of extracellular polysaccharides, and presence of certain enzymes **(Rattanachaikunsopon and Phumkhachorn, 2010; Konig *et al*., 2017).**

More recently, genetic techniques such as 16S rDNA sequencing have been developed which allows a more consistent and accurate identification of individual strains **(Khalisanni, 2011).** LAB belong to the phylum Firmicutes, Class Bacilli, and order Lactobacillales whereas Bifidobacterium belongs to Actinomycetes **(Siezen *et al*., 2002; Mokoena, 2017).** And they are divided to six genera **(Table I.1).**

Table I.1: Current taxonomy of lactic acid bacteria (Mozzi *et al*., 2016).

Family	Genus
Aerococcaceae	*Abiotrophia*, *Aerococcus*, *Dolosicoccus*, *Eremococcus*, *Facklamia*, *Globicatella*, *Ignavigranum*.
Carnobacteriaceae	*Alkalibacterium*, *Allofustis*, *Alloiococcus*, *Atopobacter*, *Atopococcus*, *Atopostipes*, *Carnobacterium*, *Desemzia*, *Dolosigranulum*, *Granulicatella*, *Isobaculum*, *Marinilactibacillus*, Trichococcus.
Enterococcaceae	*Enterococcus*, *Melissococcus*, *Tetragenococcus*, *Vagococcus.*
Lactobacillaceae	*Lactobacillus*, *Paralactobacillus*, *Pediococcus*.
Leuconostocaceae	*Leuconostoc*, *Oenococcus*, *Weissella*.
Streptococcaceae	*Lactococcus*, *Lactovum*, *Streptococcus*.

I.1.4. Probiotic bacteria

Due to their health benefits, some LAB are used as probiotics. The term probiotics is derived from the Greek "probios" which means "for life" **(Florou-Paneri, 2013; Mokoena, 2017).** Probiotics are defined by the World Health Organization as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" **(FAO/WHO, 2001).** Some of these health benefits are : immune stimulation, cholesterol reduction, inhibition of pathogen growth, maintain of a healthy gut microflora, prevention of cancer, improvement in lactose utilization, prevention of diarrheal diseases or constipation, absorption of calcium and synthesis of vitamins and predigestion of proteins **(Yan Li *et al*., 2009; Khosravi Zanjani *et al*., 2014).**

Their efficacy depends on the dose level and their viability throughout storage, product shelf life and their survival in gut environment **(Sathyabama *et al*., 2014)**. **U**nfortunately, most of the probiotics lack the ability to survive in high quantity because of low pH in gastric juice, exposure to oxygen and bile concentration that limited their effectiveness **(Ding and Shah, 2007; Shori, 2017).**

In the past, species from LAB have been used as probiotic such as *E. faecium*, *S. thermophilus*, *Le. mesenteroides*, *L. lactis* subsp. *lactis*, *E. faecalis* and, *P. acidilactici*. Probiotics belong to the genera of *Lactobacillus* and *Bifidobacterium* used for human have been isolated from the human gastrointestinal tract **(Dhamale *et al*., 2015).**

I.1.5. Application of lactic acid bacteria

LAB are widely used as starter cultures in the food industry for the production of fermented foods, including dairy products (yogurt, cheese), meat (sausages), fish, cereals (bread and beverages such as beer), fruit (malolactic fermentation processes in wine production), and vegetables (sauerkraut, kimchi, silage) **(Calo-Mata *et al*., 2008).**

They are also used for maintaining the nutritive quality and improving the shelf life of foods by inhibiting the growth of pathogenic and deteriorating microorganisms producing antagonistic substances such as bacteriocins. They have also been used as flavor and texture producers **(Parada *et al*., 2007). Table I.2** bellow summarizes the field of application of the genera of LAB.

Table I.2: The genera of LAB and their applications (Dhamale *et al*., 2015).

Genera	Application
Lactobacillus, Leuconostoc	Food industry
Carnobacterium, Pediococcus	Bacteriocin production
Aerococcus, Vagococcus	Biopharmaceutical industry
Weissella, Enterococcus	Food and health industry
Streptococcus, lactococcus	Diary industry

I.2. Microencapsulation technology

I.2.1. Definition of encapsulation

Encapsulation is a physicochemical or mechanical process in which tiny particles, droplets or gas compounds are surrounded by a coating or embedded in a homogeneous or heterogeneous matrix to give small capsules that release their contents at controlled rates under the influences of specific conditions over prolonged periods of time (**Anal and Singh, 2007; Champagne and Fustier, 2007; Burgain *et al*., 2011)**. Microencapsulation can provide a physical barrier between the core compound and the other components of the product that inhibits chemical interactions; and thus, protects against the effects of environmental factors (e.g., temperature, pH, enzymes, and oxygen) **(Poshadri and Kuna, 2010; Dias *et al*., 2017).**

From microbiological point of view, microencapsulation can be defined as the process of entrapment/enclosure of microorganisms cells by means of coating them with proper hydrocolloid(s) in order to separate the cells from the surrounding environment to reduce cell injury or cell loss in a way that results in appropriate cell release in the intestinal medium (**Krasaekoopt *et al*., 2003; Mortazavian *et al*., 2007).**

I.2.2. Structure and types of microcapsules

I.2.2.1. Structure of microcapsules

The microcapsules are generally spherical, ovoid, and even irregular and might have even/smooth or rough surfaces which have a size ranging from 1µm to 1mm **(Lakkis, 2016).** It consists of a semipermeable, spherical, thin, and strong membrane surrounding a solid/liquid core

(Anal and Singh, 2007). In fact, both the size and shape of formed microparticles depend on the materials and methods used to prepare them **(Poshadri and Kuna, 2010).** The encapsulated substance called the core material is dispersed in a matrix also named coating or shell **(Burgain *et al*., 2011)**. The core may be composed of just one or several ingredients and the wall may be single or double-layered in order to increase microencapsulation efficiency. The retention of these cores is governed by their chemical functionality, solubility, polarity and volatility **(Poshadri and Kuna, 2010).**

I.2.2.2. Types of microcapsules

These microparticles are made up of polymeric materials that can entrap active particles at various concentrarions. The active particles are made up of drugs, perfums, flavors, vitamins, pigments or dyes, minerals, probiotics and other biological materials **(Lakkis, 2016).** Emulsification generates oily or aqueous droplets commonly named capsules, while the extrusion gives gelled droplets called beads. The core of the capsule is liquid while the core of the bead presents a porous network **(Gbassi and Vandamme, 2012).**

Two main types of capsules might be distinguished

- The reservoir type; has a shell around the core material and this is why it can also be called a capsule, this type is also called, single-core, mono-core or core-shell type.
- The matrix type; the active agent is dispersed over the carrier material, it can be in the form of relatively small droplets or more homogenously distributed over the encapsulate and also present at the surface.

A combination of these two types gives a third type of capsule: coated matrix where the active agent is recovered by coating **(Figure I.1) (Zuidam and Nedovic, 2010; Burgain *et al*., 2011).**

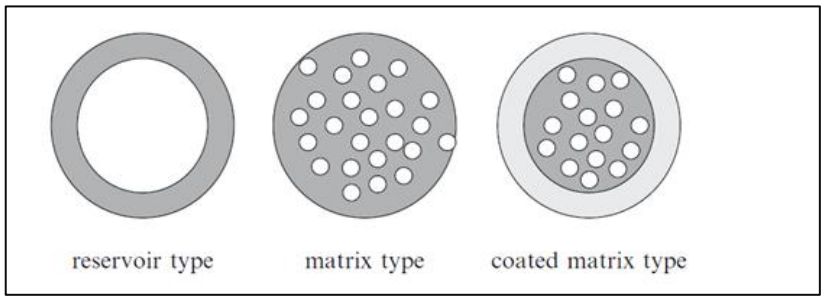

Figure I.1: Types of capsules (Zuidam and Nedovic, 2010).

I.2.3. Methods of microencapsulation

Various techniques for microencapsulation of microbial cells have been investigated over the past few years for the protection and viability enhancement of microorganisms, (**Rathore *et al*., 2013; Đordevic´*et al*., 2014).**

The choice of the most suitable encapsulation method essentially depends on the type of core material and the characteristics of the final product where the encapsulation will be applied, also the application of the microspheres in order to guarantee the survival of bacteria during the encapsulation process, in storage conditions and consumption, as well as the controlled release in the specific desired area of gut. It is essential that the encapsulation process is performed under relatively mild conditions to ensure high viability of the encapsulated cells **(Chávarri *et al*., 2012; Rathore *et al*., 2013; Dias *et al*., 2017).**

The selected method should be able to produce microspheres with the necessary physical/chemical attributes while causing minimal damage to cell integrity and viability and be easy to scale up with acceptable processing costs **(Rathore *et al*., 2013).**

I.2.3.1. Extrusion method

Extrusion is the oldest and the most commun technique, it is a physical technique used for micrencapsulating bacteria in hydrocolloid gel matrices (alginate and carrageenan) due to its ease; simplicity; low cost; and gentle conditions, which ensure high retention of the microencapsulated bacteria **(Lakkis, 2016; Burgain *et al*., 2011)**.

Technically, the process involves preparing a hydrocolloid solution, adding micro-organisms to it, and extruding the cell suspension through a syringe needle in the form of droplets to free-fall into a hardening solution or setting bath **(Figure I.2)**. The size and shape of the beads depend on the diameter of the needle and the distance of free-fall, respectively, as well as surface tension of the hardening solution. This method has been used for producing beads with 2 to 5 mm diameters **(Krasaekoopt *et al*., 2003; Lakkis, 2016).**

Extrusion technologies have many advantages for encapsulation of microbes. It is relatively gentle, does not involve deleterious solvents, does not need organic solvents and it is easy to control the size of beads by varying the applied potentiel, and can be done under both aerobic and anaerobic conditions, this latter is especially advantages when anaerobic microorganisms are being applied in food products. Extrusion technologies are also applied for flavors, enzymes, and proteins **(DeVos *et al*., 2010; Martín *et al*., 2014).**

The most important disadvantage of this method is that it is difficult to use in large scale productions due to the slow formation of the microbeads (**Burgain *et al*., 2011).**

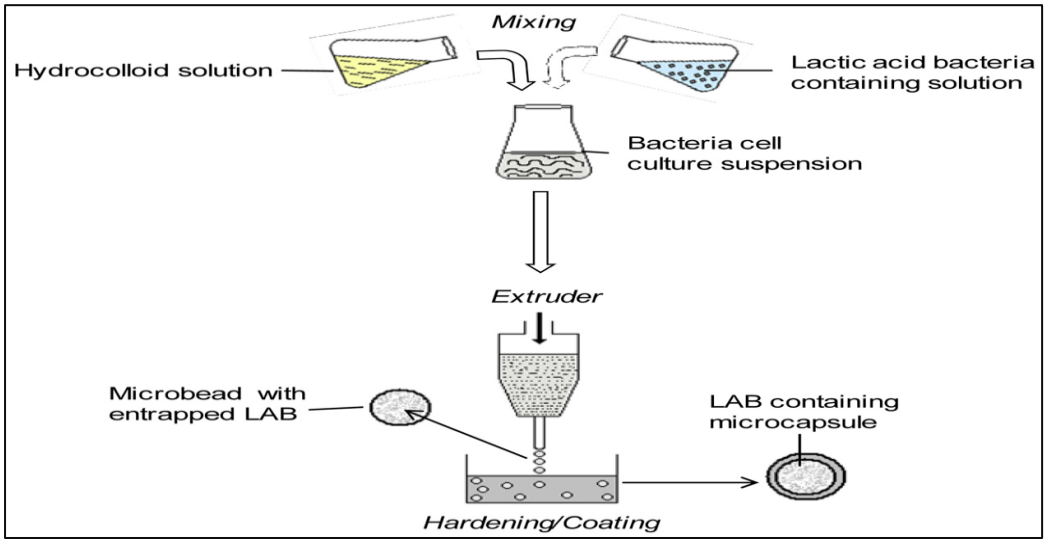

Figure I.2: Scheme of extrusion procedure (Feucht and Kwak, 2013).

I.2.3.2 Emulsification method

Encapsulation by emulsion technique was developed in the early 1980 to immobilize sensitive living cells and it is hence certainly one of the most applied methods to generate LAB containing microcapsules until today, it is a chemical technique based on the interaction between the continuous and discontinuous phases **(Feucht and Kwak, 2013; Cheng, 2015).** In this technique, the discontinuous phase (cell polymer suspension) is added to a large volume of oil (continuous phase) such as soyabean, sunflower, canola or corn oil, some studies have used white light paraffin oil and mineral oil **(Figure I.3)**. Emulsifiers are also added to form a better emulsion. Tween 80 at the concentration of 0.2% has been recommended as the best choice, because the emulsifiers lower the surface tension, resulting in smaller particle **(Martín *et al*., 2014).**

The mixture is homogenized to form a water in oil emulsion. Once the water in oil emulsion is formed, the water-soluble polymer is insolubilized (cross-linked) to form the particles within the oil phase which is then separated by filtration or centrifugation **(Favaro-Trindade *et al*., 2011).**

This method can easily be scaled up and generate small beads with a diameter of around 25 µm to 2 mm. It has been reported that concentration and viscosity of the encapsulation mix before gelation and its agitation rate and type of emulsifier are the main parameters that control the diameter of the final formed microbeads **(Huq *et al*., 2013).**

Emulsification is more expensive because it requires additional raw materials such as vegetable oil and emulsifiers to stabilize the emulsion. It also presents difficulties in implementation including emulsion instability, need for vigorous stirring which can be detrimental to cell survival, random incorporation of cells into the capsules, and inability to sterilize vegetable oil if you have to work under conditions of strict asepsis **(Gbassi and Vandamme, 2012)**.

The combination of processes can lead to promising results. **Picot and Lacroix, 2004** combined emulsification with spray drying to develop an encapsulation method on a large scale. In this work, the powder containing the probiotic bacteria was added to milk fat (dispersed and oil phase) which was emulsified in a solution of serum isolated protein (aqueous and continuing phase) to form w/o emulsion then the material was spray dried **(Garti, 2008).**

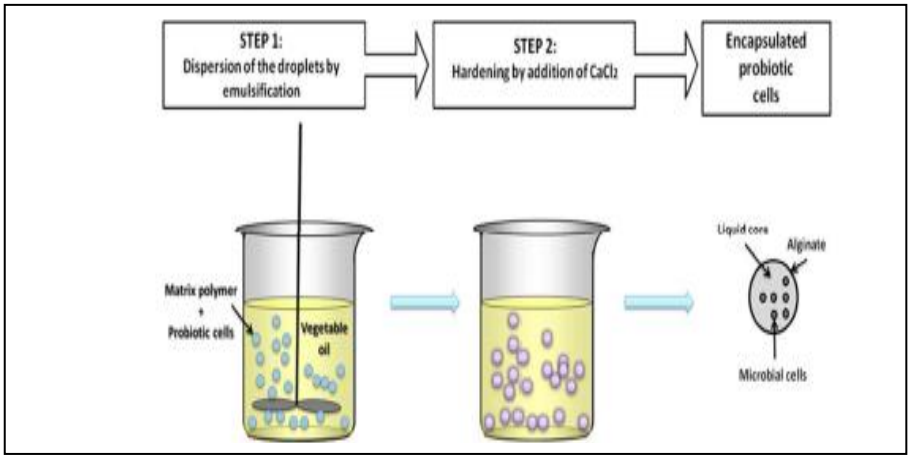

Figure I.3: Scheme of emulsification procedure (Burgain *et al*., 2011).

I.2.3.3. Drying methods

I.2.3.3.1. Spray drying

Spray drying is one of the oldest encapsulation methods used since the 1930 to prepare the first encapsulated flavors using gum acacia as wall material. In food industry, spray drying is a commonly applied encapsulation method producing large amounts of microcapsules in one continuous process step. This method is very suitable when microencapsulated probiotics need to be dried in order to allow storage over a long period **(Gharsallaoui *et al*., 2007; Feucht and Kwak, 2013)**.

The process involves the dispersion of the core material into a polymer solution, forming an emulsion or dispersion, followed by homogenisation of the liquid, then atomisation of the mixture into the drying chamber **(Figure I.4)**. This leads to evaporation of the solvent (water) and hence the formation of matrix type microcapsules **(Kailasapathy, 2002).** The core material retention during microencapsulation by spray-drying is affected by the composition and the properties of the emulsion and by the drying conditions **(Gharsallaoui *et al*., 2007).**

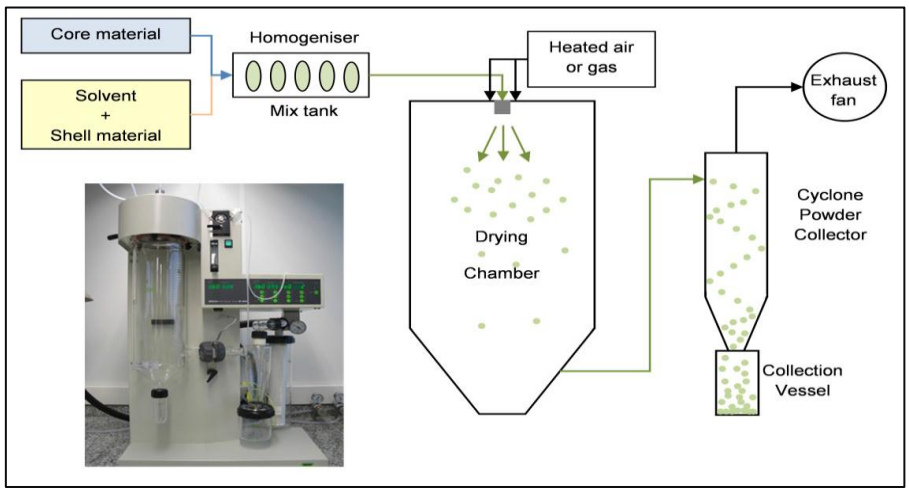

Figure.I.4: Scheme of spray drying procedure and image of a mini spray dryer (Chávarri *et al*; 2012).

I.2.3.3.2. Lyophilization method (freeze drying)

Lyophilsation is a process used for the dehydration of almost all heat sensitive materials and aromas. Freeze-drying has been used to manufacture probiotic powders for decades but the combination of freeze drying and encapsulation is relatively new concept **(Desai and Park, 2005; Martín *et al*., 2014).**

It is performed by the sublimation of ice of the frozen sample under vacuumat absolute pressure between 0.05 to 0.1 mBar and temperature between -50 to -30°C **(Cook *et al*., 2005; Cock and Castillo, 2012).** Once lyophilized, cryoprotectants are added to preserve and stabilize the probiotic activity during storage, the most common cryoprotectans are lactose, trehalose, sorbitol, sucrose, milkprotein and skim milk, they are able to accumulate within the cells, reducing the osmotic difference between the internal and external environments **(Cock and Castillo, 2012; Martín *et al*., 2014).**

Freeze drying is a very expensive technology 4 to 7 time than spray drying need a high energy and long processing time, in addition freezing causes damage to the cell membrane because of crystal formation and also imparts stress condition by high osmolarity **(Chávarri *et al*., 2012; Martín *et al*., 2014).**

I.2.3.3.3 Spray chilling or cooling method

Spray-chilling or spray-cooling is another technology to produce lipid-coated active agents, it consist of making a dispersion of a matrix and the bioactive product but instead of evaporating the dispersion is cooled by the injection of cold air (with a temperature below the melting point of the lipid) into a chamber and their atomization through a pneumatic nozzle into a vessel in order to

solidify the gel particles, the size of the particles depends on the core particles, melt viscosity, melt temperature, disk configuration and the rotational speed **(Kailasapathy, 2009; De Vos *et al*., 2010; Zuidam and Nedovic, 2010; Dias *et al*., 2017).** Spray chilling is considered to be the least expensive encapsulation technology and offers a few advantages over other encapsulation techniques. The disadvantage of spray chilling and spray cooling is that special handling and storage conditions are required **(Kailasapathy, 2009).**

I.2.3.4. Coacervation method

Coacervation is a modified emulsification technology, it consists of three steps comprising of phase separation, deposition and solidification **(DeVos *et al*., 2010; Nag, 2011).** The first step includes the formation of three immiscible phases; liquid manufacturing vehicle, core material and coating material (**Venkata Naga Jyothi *et al*., 2010)**.The second step includes deposition of liquid polymer upon the core material. Finally, the prepared microcapsules are stabilized by cross-linking, desolvation, thermal treatment or enzymatic methods. The formed microparticles are then collected by filtration or mild centrifugation followed by drying **(Nag, 2011).**

The size of the capsule and its characteristics can be varied by changing the pH, the ion concentration, the ratio of matrix molecule and the bioactive component, and the type of matrix **(DeVos *et al*., 2010).** The process can be identified according to the number of polymer type(s).The simple method is the evaporation of the solvent surrounding the molecules of a colloid with the addition of other non-electrolyte solvent (e.g. salts or alcohols) in which the colloid is insoluble. The complex coacervation is the combination of two oppositely charged hydrocolloid solutions, causing interaction and precipitation of complex polymers **(Zuidam and Nedovic, 2010; Favaro-Trindade *et al*., 2011).**

Compared with others methods, Coacervation is a promising microencapsulation technology because of the very high payloads achievable (up to 99%) and because it doesn't need high temperatures or organic solvents and allow the incorporation of a large number of microorganisms in relation to the encapsulant **(Chávarri *et al*., 2012; Gouin, 2004; Favaro-Trindade *et al*., 2011).**

I.2.3.5. Liposome entrapment

Liposomes are spherical bilayers which enclose bioative molecules, it is formed by dispersion of polar lipids (mostly phospholipid) in aqueous solution**,** due to their structure, they are able to encapsulate both water-soluble and lipid-soluble bioactives as well as amphiphilic compounds, which make them suitable for the encapsulation of wide array of bioactive compounds **(De Vos *et al*., 2010; Trifković *et al*., 2016).**

I.2.3.6. Molecular inclusion

This method involves entrapment of smaller molecules inside the hollow cavity of a larger molecule. Cyclodextrins are commonly used but restricted in the certain countries. Cyclodextrins (CD) are a group of natural oligosaccharides containing six, seven or eight glucose residues, inter linked byα (1→4) glycoside bonds in a structure shaped like cylinder. The most important advantage of CD is that it enhances the solubility of poorly water-soluble bioactives, in addition, it was proved that β – CDs are highly heat stable, can tolerate up to 200°C, and are highly resistant to chemical degradation (**Solanki *et al*., 2013; Trifković *et al*., 2016).** Some of the major limitations of this molecular inclusion technology are low payload and high cost of raw material **(Solanki *et al*., 2013).**

I.2.3.7. Adhesion to starch method

Adhesion property to starch by bacteria has been applied for the microencapsulation of probiotics. Thus, starch granules can be used as vehicles for their administration into the food. For the production of starch microcapsules, the granules are inoculated with the bacterial culture, fermented, and dehydrated **(Favaro-Trindade *et al*., 2011).**

A variety of starches and modified starches have been tested like: a calcium induced alginate polymer containing Hi-Maize™ starch, Hylon VII maize starch granules and the use starch granules combined with amylose coating **(Rokka and Rantamäki, 2010).** The disadvantage of this method is that the obtained particles affecte food texture **(Favaro-Trindade *et al*., 2011).**

I.2.3.8. Rennet-gelled protein encapsulation

The process is based on enzymatic gel formation of the encapsulating material and the subsequent application of one of the conventional encapsulation technologies **(Cock and Castillo, 2013).** Microcapsules can be produced using a food approved enzyme (rennet) and an aqueous milk protein solution **(Figure I.5)**. Cleavage of k-caseine by rennet produces the aggregation of the casein micelles, non-covalent cross-links are then progressively formed between chains of flocculating micelles to form a final gel. These microcapsules are able to encapsulate probiotics, without significant loss of cells during the encapsulation process **(Martín *et al*., 2014).**

Survival of encapsulated cells can probably be explained by a higher local pH value within the protein matrix of the capsules caused by the protein buffering capacity. It can protect the cells during incubation under simulated gastric conditions at low pH. Furthermore, these proteins alleviate the feasibility to control the capsule size of microcapsules, which is of high importance regarding the sensory impact of the particles in final products food **(Vidhyalakshmi *et al*., 2009).**

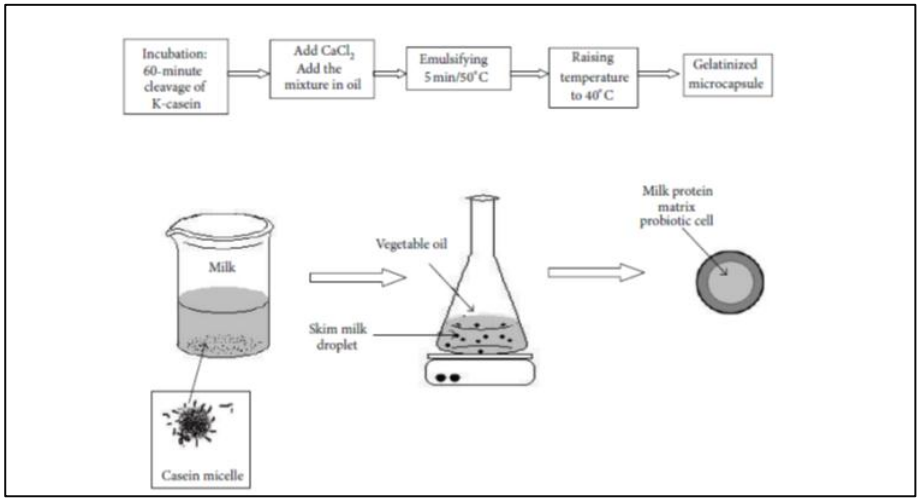

Figure I.5: Rennet gelation of milk proteins procedure (Solanki *et al*., 2013).

I.2.4. Factors affecting microencapsulation process

Different factors affecting the microencapsulation are discussed below:

- Effect of various biomaterials on viability of probiotics

Many biomaterials were tested to check their effects on the process of microencapsulation and the viability of probiotic bacteria **(Solanki *et al*., 2013).**

- Capsule characteristics with respect to the surrounding environment

Selection of the material respecting the surrounding environment is very important. microcapsule formed by alginate and different combination contribute to calcium ions leakage from alginate capsule structure leading to its decomposition. All the capsules must be resistant to the acidic conditions of gastric juices (selection of capsule material take in account the time of decomposition after subjecting them to gastric conditions) **(Solanki *et al*., 2013).**

- Coating of the capsule

Coating of capsules is an efficient way to improve their physicochemical characteristics. For example, shell coating on the alginate capsules makes them resistant to the chelating agents of calcium ions **(Mortazavian *et al*., 2007).**

- Concentration of capsule making solution and bead diameter

Encapsulation efficiency has affected by concentration of capsule making solution and final bead diameter. Protective effects against the violent environmental factors increase whith the increase of beads diameters **(Mortazavian *et al*., 2007; Solanki *et al*., 2013).**

- Environmental conditions

Physiology of the gastrointestinal (GI) tract is important during the probiotic encapsulation process where environmental factors could reduce encapsulation effectiveness **(Solanki *et al*., 2013).**

- Modification of capsule materials

Chemical modification of encapsulating material (by direct structural changes and/or addition of special additives) improves encapsulation effectiveness **(Solanki *et al*., 2013).**

- Initial concentration of microbial cells

Efficiency of encapsulation increases whith the increase of microbial cell concentration **(Solanki *et al*., 2013).**

- Conditions of processing factors

Microencapsulation processes such as freeze drying, spray drying, micronization, and storage conditions are employed in order to avoid injuries to the beads and contained cells. Also process factors can influence beads diameter **(Solanki *et al*., 2013; Mortazavian *et al*., 2007).**

- Miscellaneous affecting factors

Other remaining factors such as mixing sequence/order of the constituents during microencapsulation, their mixing proportion and mechanical tensions which might make crackling or fracturing of the beads can also affect effectiveness of microencapsulation **(Mortazavian *et al*., 2007).**

I.2.5. The advantages and disadvantages of encapsulation

Shahidi and Han (1993) proposed six reasons for applying microencapsulation in food industry:

- to reduce the core reactivity with environmental factors;
- to decrease the transfer rate of the core material to the outside environment;
- to promote easier handling;
- to control the release of the core material;
- to mask the core taste
- and finally to dilute the core material when it is required to be used in very minute amounts
- stabilizing the core material,
- controlling the oxidative reaction,
- masking colours or odours,
- extending the shelf life and protecting components against nutritional loss,
- avoids the formation of harmful and undesirable compounds as a result of chemical changes over time.

Moreover, the encapsulation of probiotics may increase microbial survival and fermentation efficiency **(Anal and Singh, 2007; Poshadri and Kuna, 2010; Dias *et al*., 2017).**

The disadvantages of encapsulation are associated with the stability of the capsule polymer matrix and the challenge of scaling up the encapsulation process and the possible negatives that can overcoming are:

- Additional costs.
- Increased complexity of production process and/or supply chain.
- Undesirable consumer notice (visual or touch) of the encapsulates in foodproducts.
- Stability challenges of encapsulates during processing and storage of thefood product **(Zuidam and Nedovic, 2010; Dias *et al*., 2017).**

I.3.Encapsulation matrices

Several biopolymer materials are available to encapsulate microbes in hydrogel matrices, depending on the desired physicochemical properties of the delivery vehicle. The most commonly used food-grade biopolymers including proteins (e.g., whey proteins and caseins and gelatin) and carbohydrates (e.g., starch and gums, carrageenan, xanthan). The choice of the capsule materials is a major element for successful microencapsulation (ME) of probiotics and the use of ME probiotics in functional foods. These microgels must be engineered to encapsulate high concentrations of probiotics and protect them from environmental stresses, such as acidic conditions pH, bile salts, and digestive enzymes **(Etchepare *et al*., 2015; Yeung *et al*., 2016).**

I.3.1. Alginate

Alginates are natural anionic polysaccharides made up of D-mannuronic and L-guluronic acid residues joined linearly by (1–4)-glycosidic linkages **(Figure I.6) (Annan *et al*., 2008).** It is linear heteropolysaccharide found in brown algae and is also produced as an extracellular matrix by certain bacteria **(Rehm, 2009)**.

Alginate has been used as the encapsulating material due to its ability to absorb water, to be easily manipulated and innocuousness, having also other features such as gelling, stabilizing and thickening, reasons which have been of great interest to the food industry. It is the most used polysaccharide as encapsulating material of lactic acid bacteria, due to ease of handling, non-toxic nature and low cost, besides increasing the viability of these bacteria when exposed to different conditions when are compared with non-encapsulated bacteria **(Etchepare *et al*., 2015).**

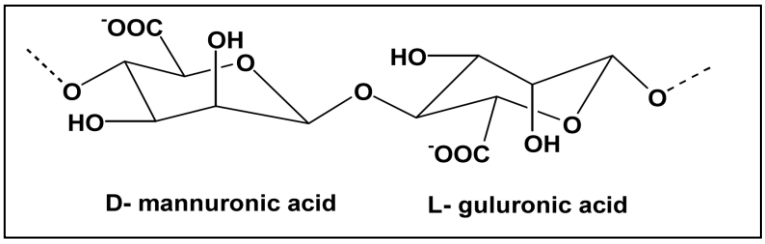

Figure I.6: Structure of alginate (Ariful *et al*., 2010).

I.3.2. Whey proteins (WP)

Whey proteins are natural vehicles for probiotics cells **(Livney, 2010)**. They are used widely as encapsulating agents in food applications due to their broad functionality including gelation and emulsification **(Heidbach *et al*., 2009; Doherty *et al*., 2011).** Moreover, for their structural and physicochemical properties they can be used as delivery system **(Heidbach *et al*., 2009)**. Encapsulated probiotic cells in these proteins based on their specificity of possessing showed excellent gelation properties **(Sarao and Arora, 2015).** Whey proteins have been used singly or in combination of several polysaccharides in microencapsulation of probiotics **(Abd El-Salam *et al*., 2015).** B-Lactoglobulin (BLG) is the main whey protein component and its principal gelling agent **(Gunasekaran *et al*., 2007).**

I.3.3. Chitosan

Chitosan is the natural cationic polysaccharide generated by alkaline deacetylation of chitin, which carries positive charges at pH values below 6.5 **(Shu *et al*., 2017).** Derived from various crustaceans and insects shells **(Cook *et al*., 2011).** Chitosan exhibited inhibitory effects on different types of lactic acid bacteria and for this reason is preferred as a coating material **(Martin *et al*., 2014).**

I.3.4. K-Carrageenan

Carrageenan is a natural carbohydrate (polysaccharide) obtained from edible red seaweeds. It is a suitable support material for the immobilization of the whole cells and is commonly used as a food additive **(Figure I.7) (Necas and Bartosikova, 2013; Chopde *et al*., 2014).**

It combined easily with milk proteins to improve solubility and texture; serve as thickening Agent, emulsifier, and stabilizer **(Necas and Bartosikova, 2013).** Carrageenan requires temperatures comprised between 40° and 50° C for dissolution especially when applied at high concentrations such as 2-5%. Gelation of carrageenan is induced by temperature changes (**Krasaekoopt *et al*., 2003)**

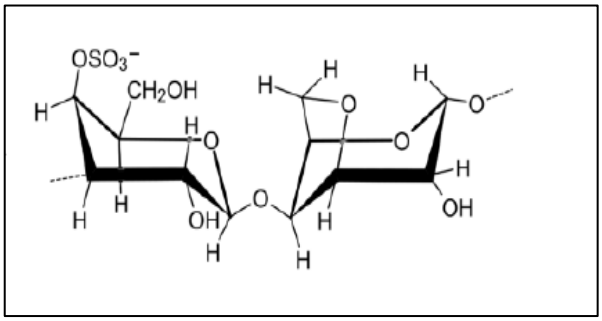

Figure I.7: Structure of K-carragenan (Chopde *et al*., 2014).

I.3.5. Cellulose acetate phthalate (CAP)

CAP is widely used as a coating agent. It is used for controlling drug release in the intestine due to its safety nature and because it is physically inert **(Mortazavia *et al*., 2008).** The advantage of this component is that it is not soluble at acidic pH (less than 5) but it is soluble at pH higher than 6 as a result of the presence of phthalate groups. The encapsulation of probiotic bacteria using CAP provides good protection for microorganisms in simulated GI conditions **(Favaro-Trindade *et al*., 2011; Chopde *et al*., 2014)**.

I.3.6. Starch

Starches are polysaccharides, composed of a number of monosaccharides or sugar (glucose) molecules linked together with a-D-(1-4) and/or a-D-(1-6) linkage. The starch consists of two main structural components, the amylose, which is essentially a linear polymer in which glucose residues are a-D-(1-4) linked, and amylopectin, which is a larger branched molecule with a-D-(1-4) and a-D-(1-6) linkages **(Sajilata *et al*., 2006**). Because of its low price, relative ease of handling and broad application starch is used as matrix of capsules for targeted delivery of a broad panel of bioactive components **(De vos *et al*., 2010).** Starch can be used to ensure the viability of probiotic populations from the food to the large intestine. Resistant starch also offers an ideal surface for adherence of the probiotics to the starch granule **(Anal and Singh, 2007).**

I.3.7. Gellan gum and xanthan gum

Gellan gum is an anionic hetero polysaccharide, secreted by microbe *Sphingomonas elodea*. It consists of glucose, rhamnose, glucuronic acid and are linked together to give a tetrasaccharide unit **(Nikode *et al*., 2016)**. Xanthan gum is an anionic polyelectrolyte consisting of a cellulosic backbone with side chains of two mannoses andone glucuronic acid on every second glucose residue **(Shu *et al*., 2017).**

I.3.8. Gelatin

Gelatin is a protein of animal origin, it is obtained by a partial hydrolysis of the fibrous insoluble collagen, which is a protein widely found in nature and is the major constituent of skin, bones and connective tissue. it is useful as reversible gelling agent for probiotic encapsulation. Because of its amphoteric nature, it also is an excellent candidate for cooperation with anionic polysaccharides like gellan gum. **(Dong *et al*., 2006; Chopde *et al*., 2014).**

ChapterII.Materials and methods

Period of work

All the manipulations of this work were conducted in the microbiology laboratory at the university Mohammed Seddik Ben yahia/Jijel during the period of April- June 2018.

II.1. Bacterial strains

Tree strains of lactic acid bacteria previously isolated and identified by Samia Amira at the laboratory of …. from traditional Algerian cheese Klila have been used: ***Lactobacillus plantarum*** (B10), ***Lactobacillus casei*** (KBM2) and ***Lactobacillus brevis*** (B1).

II.2. Media and buffers

- MRS medium (Man Rogosa Sharp) broth and agar.

- Buffer solution (PBS).

- Normal saline solution (0.9%).

II.3. Polymers

- Sodium alginate polymer (2%).

- K- carrageenan- alginate polymer (1%- 1%).

- Prebiotic polymer (alginate 2%- glycogen 1%- glycerol 2.5%).

- Alginate – chitosan polymer (2%- 0.8%).

II.4. Apparatus

- Balance (Scout Pro)

- pH meter (HANNA instrument)

- Spectrophotometer (Amersham Biosciences)

- Optical microscope (Paralux)

- Vortex (Minishaker IKA)

- Autoclave (Slli AVX electronic)

- Centrifuge (HETTICH ZENTRFUGEN)

- Water bath

- Incubator (Mammert)

II.5. Preparation of bacterial culture

Stored cultures of ***Lactobacillus plantarum*** (B10), ***Lactobacillus casei*** (KBM2) and ***Lactobacillus brevis*** (B1) isolated from Klila cheese, previously purified and identified as mentioned above, have been used.

A check for purity before the use has been conducted with macroscopic and microscopic tests of catalase assay, the observation of colonies with same color, shape and size and Gram staining. These pure strains were inoculated into 10 ml MRS broth and incubated at 37°C for 24h under aerobic conditions. The cells were harvested by centrifuging at 6000 rpm/ 15 min washed once by 0.9% NaCl (10 ml) and suspended in 10 ml of normal saline solution NaCl (0.9%). The resulting cell suspensions were used directly for assessing the survival of free cells or subjected to encapsulation as described in section "Microencapsulation ".

II.6. Microencapsulation of bacterial strains in different biopolymers

The extrusion method described by **De prisco *et al*. (2015)** with some modifications was adopted for encapsulation of ***Lb. plantarum***, ***Lb. casei*** and ***Lb. brevis*** cells.

the microencapsulation was carried out using 2% sodium alginate, 1% alginate with 1% K-carrageenan, and 2% alginate with 1% glycogen solution previously autoclaved (121°C for 20min) the previously obtained cell suspension was aseptically microencapsulated by mixing it with polymer solution (40ml) to obtain a final volume of 50ml. The mixture containing bacterial cells were introduced into a sterile syringe (2,5ml) and dropped and hardened in 200 ml of a 0.5 mol/L calcium chloride ($CaCl_2$) solution (in ratio of 4:1 with polymer cell suspension) previously autoclaved and cooled.

The resulting beads formed were then left for 45 min with rotation at room temperature, filtered and then washed with sterile distilled water. Finally, the beads were conserved in normal saline at 4°C for further utilization. Free cells were managed in the same way and used as control sample.

II.7. Double coating of alginate microcapsule with chitosan

The chitosan solution was prepared based on the method described by **Jantarathin *et al*. (2017)** with some modifications. Chitosan solution was prepared by dissolving 8 g of chitosan in 1000 mL of distilled water. The pH was adjusted to 3.2-3.4. The solution was autoclaved at 121°C for 20 min. The alginate beads were transferred to 100 ml of 0.8% chitosan solution. The microcapsules were stirred gently with a magnetic bar for 15 min to ensure the evenly coated of the surface of the alginate bead. Such microcapsules were then separated by filtration, then rinsed with distilled water.

II.8. Effect of storage at different temperature on the viability of free and encapsulated cells:

The evaluation of the viability of encapsulated and free cells was realized by the addition of 1g of beads into 9ml of physiological normal saline and their incubation under various temperatures 0°C, 4°C and 25°C during 28 days

II.9. Effect of the storage in the fruit juice on the viability of free and encapsulated cells:

Commercial pineapple juice N'gaous (the composition is in the appendices) purchased from local supermarket was used for the storage of encapsulated (E) cells. According to the method of **Nualkaekul *et al*., (2013)** and **Nualkaekul *et al*., (2011),** 1g of beads of each polymer prepared as described in section II-6 was added to 9 ml of juice in sterile tube for all strains. The juice was stored at 4°C for 04 weeks. Samples were collected weekly and analyzed for cell viability.

II.10. Simulated gastrointestinal (GI) conditions

Simulated gastric and enteric juices were prepared according to **Brinques *et al*. (2011)** and **Deprisco *et al*. (2014)**. Simulated gastric solution (SGS) was prepared by adding 3g/L pepsine (1038 U ml^{-1}) to the gastric solution at pH 2. Simulated intestinal solution (SIS) was prepared by suspending pancreatin in the intestinal solution to a final concentration of 1g/l, with 5g/l bile salts and adjusting the pH to 7 with sterile 0.1 mol/L Na OH. Both solutions were sterilized by filtration (0.22 µm).

Two series of tubes are used, 1g of encapsulated cells of each polymer was suspended in 9 ml of simulated gastric solution (SGS) for the both series than incubated at 37°C. after the period of incubation, beads of the first series used for the counting of the number of cells, beads of the second series of tubes submitted to the SIS then incubated at 37 °C with shaking by hand each 30 min. After this period bacterial cells enumerated using serial dilution and count by Mallassez cell. Free cells were managed in the same way and used as control sample.

II.11. Release of encapsulated cells:

To determine the viable counts, the entrapped bacteria were released from the beads according to the method of **Sultana *et al*., (2000)** and **Brinques *et al*., (2011**), One gram of beads was re-suspended in 9 ml of phosphate buffer (0.5 M, pH 7.4) or sodium citrate 5% followed by homogenizing in a vortex 10 min. The formed solution was then used to determine the number of viable cells using serial dilution and counted by MALLASSEZ cell each week.

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ChapterIII.Results and discussion

III.1. Characterization of beads

The beads sizes were determined by measuring diameters with a calibrated micrometer scale and the average weight of four (4) beads of each polymer was taken and the results were showed in the tables **Table III 1.**

Table III.1: General characteristics of bacterial beads.

Strains / Polymers	*Lb. casei*		*Lb. brevis*		*Lb. plantarum*	
	Diameter	Weight	Diameter	Weight	Diameter	Weight
Alginate	1.52	5.92	1.59	7.82	0.99	1.92
Alginate-chitosan	1.39	5.12	1.54	5.13	0.74	3
k-carrageenan	2.25	15.5	2.3	9.9	2.03	9.75
Alginate-glycogen	1.6	7.9	1.61	7.51	1.14	4.55

The tables above demonstrate that beads diameters are different depending on the bacterial strain and the polymer of encapsulation. The average diameter of alginate beads is ranged between 0.99 mm and 1.59 mm, whereas the beads of alginate coated with chitosane showed the lowest diameter (0.74 mm). The k-carrageenan beads were the largest ones with diameter of 2.3 mm, while beads of alginate and alginate-glycogen showed near diameters.

III.2. Aspect of encapsulated bacterial strains

The shape of the beads obtained after the microencapsulation procedure was generally spherical; sometimes elliptical shaped beads were observed. The alginate beads and the alginate-glycogen beads had a rounded smoother surface for the ***Lb. casei*** and ***Lb. brevis*** strains of bacteria whereas ***Lb. plantarum*** beads had irregular smaller shape. The k- carrageenan beads had an elliptical and irregular shape and they appear as the largest beads **(Figures III.1, III.2 and III.3).**

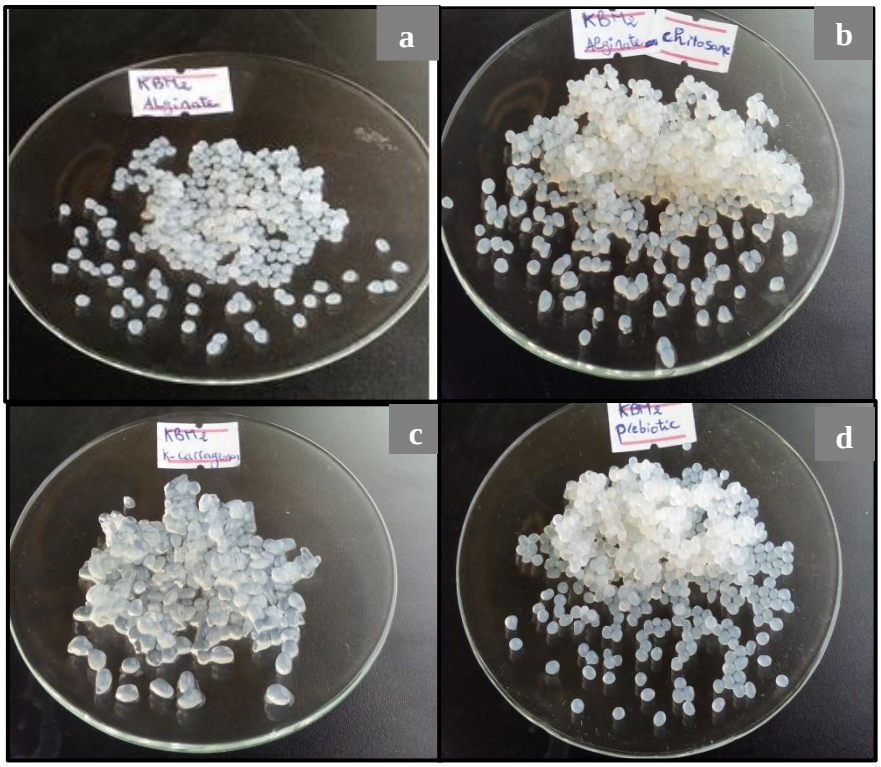

Figure III.1: **Aspect of encapsulated *Lb. casei* (KBM2): (a) beads of alginate, (b) beads of alginate coated with chitosan, (c) beads of k-carrageenan, (d) beads of alginate-glycogen.**

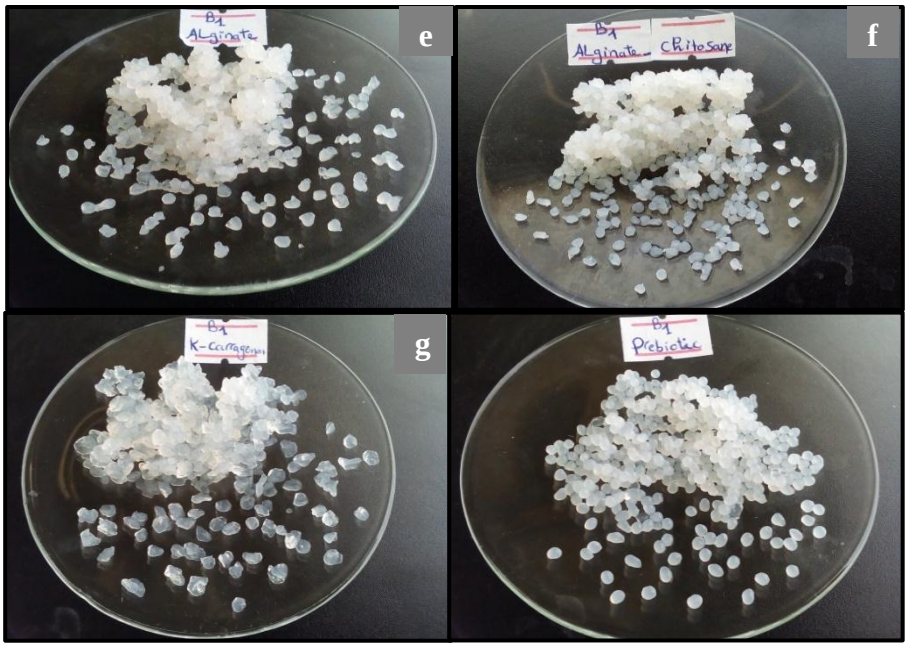

Figure III.2: Aspect of encapsulated *Lb. brevis* (B1): (e) beads of alginate, (f) beads of alginate coated with chitosan, (g) beads of K-carrageenan, (h) beads of alginate-glycogen.

Figure III.3: Aspect of encapsulated *Lb. plantarum* (B1): (i) beads of alginate, (j) beads of alginate coated with chitosan, (k) beads of k-carrageenan, (l) beads of alginate-glycogen.

Compared to alginate beads, the chitosan-coated beads had jagged edges, according to **(Yeung *et al*., 2016)** this observation suggests that the chitosan layer has been successfully deposited onto the external surfaces of the alginate microgels. In addition, **Fareez *et al*., (2015)** found that the irregularity shape reflects higher polymer concentration on the surface of the beads.

In this work, in spite we used the same concentration of polymer (2%) according to **(Chávarri *et al*., 2010),** there were different beads sizes among different kinds of beads because they had different amount of probiotic inside.

III.3. Effect of storage at different temperatures on the viability of free and encapsulated cells

These experiments were performed in order to evaluate the efficiency of encapsulation to reduce the loss in number of the bacterial strains (free and encapsulated cells) under different temperatures and the results were represented in the figures below.

The initial cell count of all bacteria before encapsulation was about 10 (1og cells/ml) to 11 (log cells/ml).

➢ ***Lactobacillus casei***

As shown in **figure III.4**, at 0°C the decrease in free cells is higher than the encapsulated cells, whereas E cells in alginate decrease from 9.22 (log cells /ml) to 7.75 (log cells/ml). At 4°C the E cells decreased from 9.40 (log cells/ml) to 8.04 (log cells/ml) and these results demonstrated a higher number than the free cells, also, at 25°C the number decreased from 9.38 (log cells/ml) to 7.86 (log cells /ml).

For the E cells in alginate coated with chitosan shown in **figure III.5**, the decrease was from 9.21 (log cells/ml) to 7.80 (log cells/ml) at 0°C, and it was from 9.39 (log cells/ml) to 8.12 (log cells /ml) at 4°C in the same storage time, whereas at 25°C, the E cells were declined from 9.23 (log cells /ml) to 8.07 (log cells/ml) in 28 days.

Figures III.6 and **III.7** show that the number of cells released from alginate-glycogen and k-carrageenan microcapsules respectively after storage. it can be seen that the number of cells released from k-carrageenan decreased during storage for 28 days, at 0°C 9.19 (log cells/ml) to 7.66 (log cells /ml), at 4°C, there was a loss of cells from 9.49 (log cells /ml) to 7.99 (log cells /ml) at the end of storage, whereas, at 25°C the number of cells decreased from 9.3 (log cells /ml) to 7.87 (log cells /ml). however, encapsulated cells in alginate-glycogen, showed a decreased from 9.1 to 7.20 (log cells /ml) at 0°C and from 9.29 to 7.98 (log cells /ml) at 4°C but at 25°C it reduced from 9.25 to 7.87 (log cells /ml).

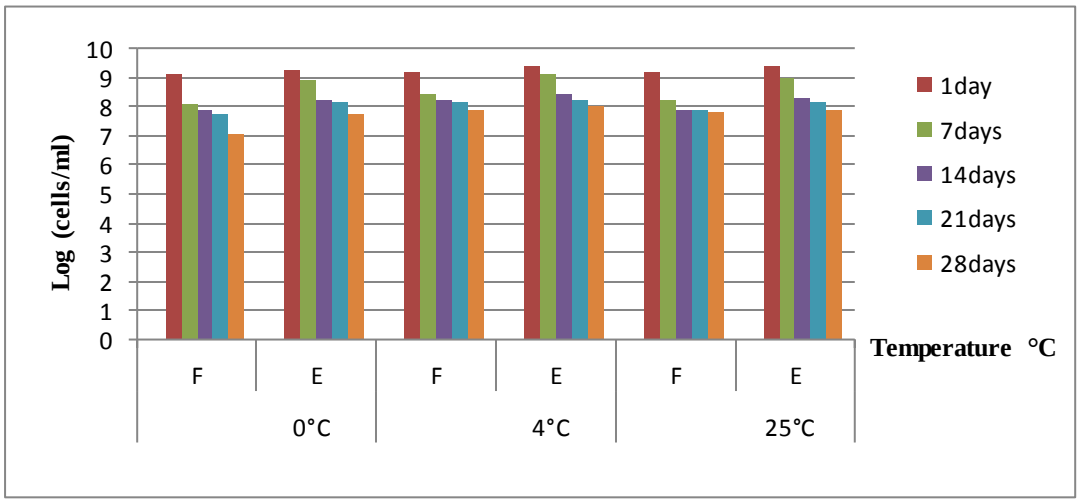

Figure III.4: Survival of free and encapsulated *Lb.casei* in alginate during storage at different temperatures.

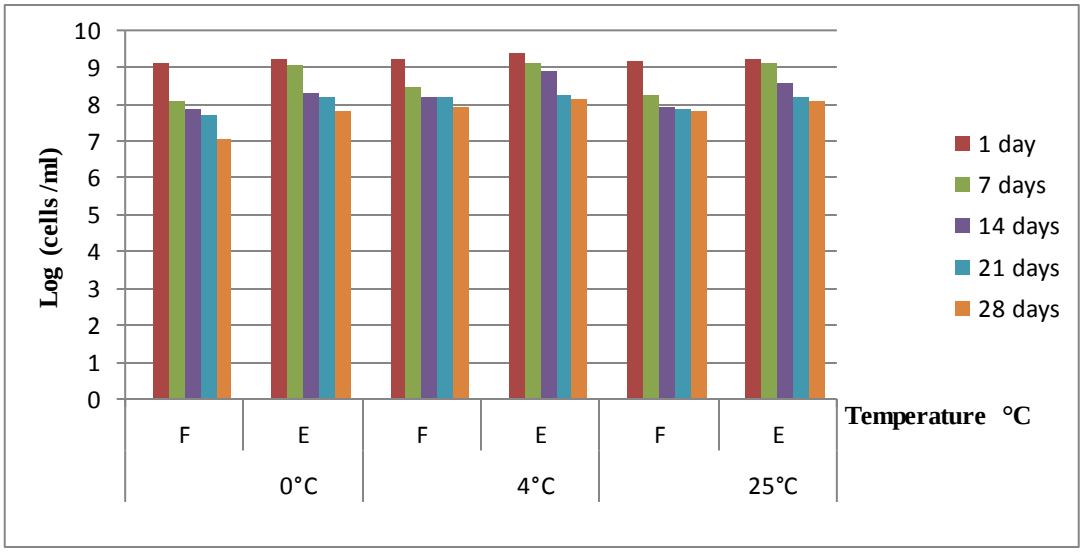

Figure III.5: Survival of free and encapsulated *Lb. casei* in alginate coated chitosan during storage at different temperatures.

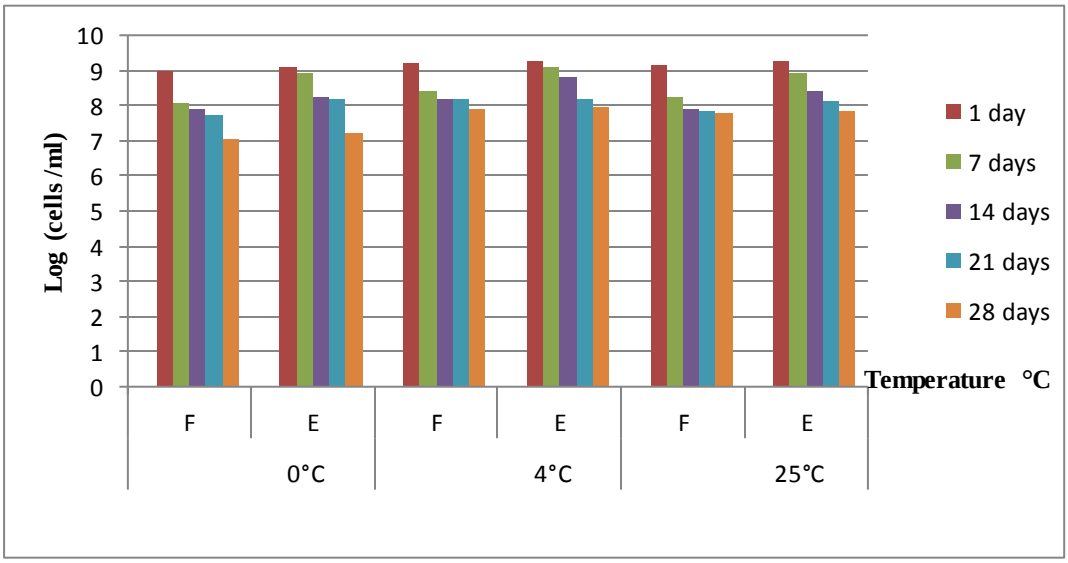

Figure III.6: Survival of free and encapsulated *Lb. casei* in alginate-glycogen during storage at different temperatures.

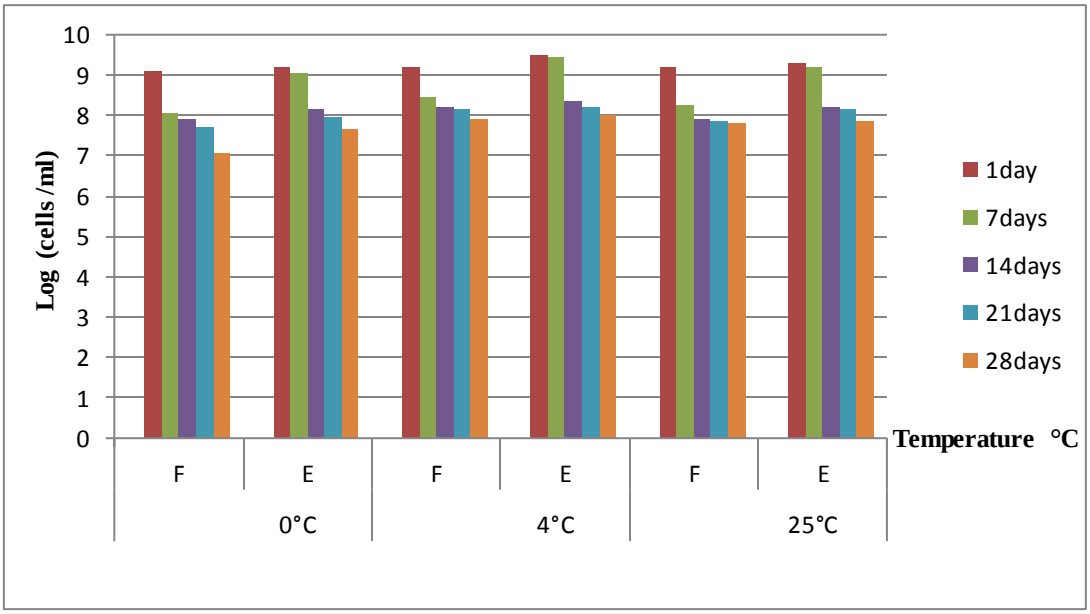

Figure III.7: Survival of free and encapsulated *Lb. casei* in k-carrageenan during storage at different temperatures.

- ***Lactobacillus brevis***

A decrease in number was observed for ***Lb. brevis*** encapsulated in all polymers of our experiment within 28 days in different temperatures of storage. **Figure III.8** show that E cells in alginate were decreased from 10.24 (log cells /ml) to 8.19 (log cells /ml) at 4°C in which the highest number was observed compared to the other temperatures at 0°C from 10.02 to 7.89 (log cells / ml), at 25°C from 10.24 to 8.07 (log cells /ml).

For alginate-chitosan encapsulation **(figure III.9)**, we found a slight decrease over the 28 days, similarly to alginate, the number at 4°C was higher than others temperatures, from10.31 to 8.29 (log cells /ml) at 4°C; from 10.15 to 7.94(log cells /ml) at 0°C; and from 10.25 to 8.19 (log cells /ml) at 25°C. according to figures **III.10** and **III.11**, the same results were observed with alginate-glycogen and K-carrageenan. However, the K-carrageenan presents better results compared to alginate-glycogen and alginate.

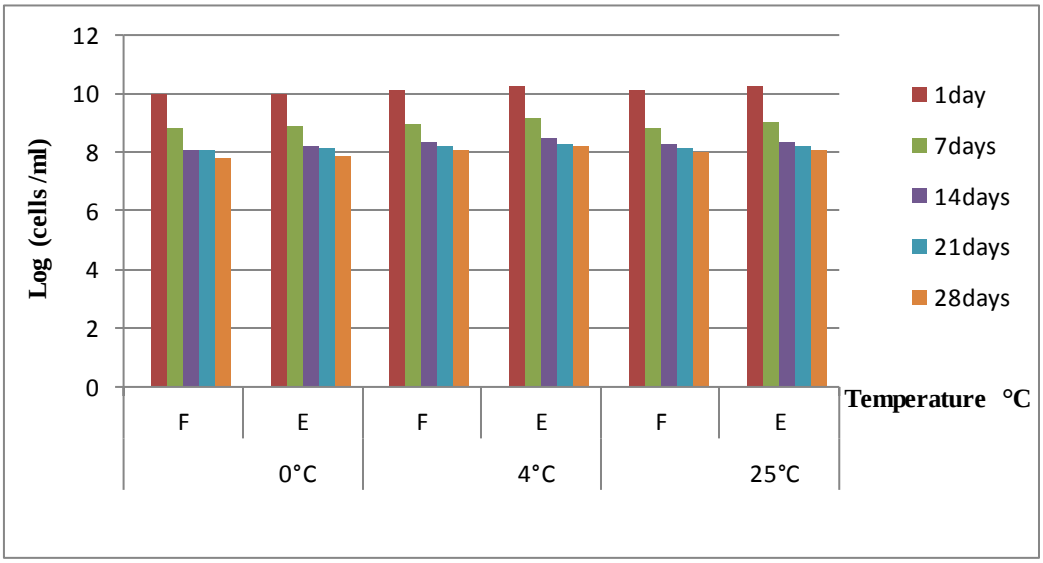

Figure III.8: Survival of free and encapsulated *Lb. brevis* in alginate during storage at different temperatures.

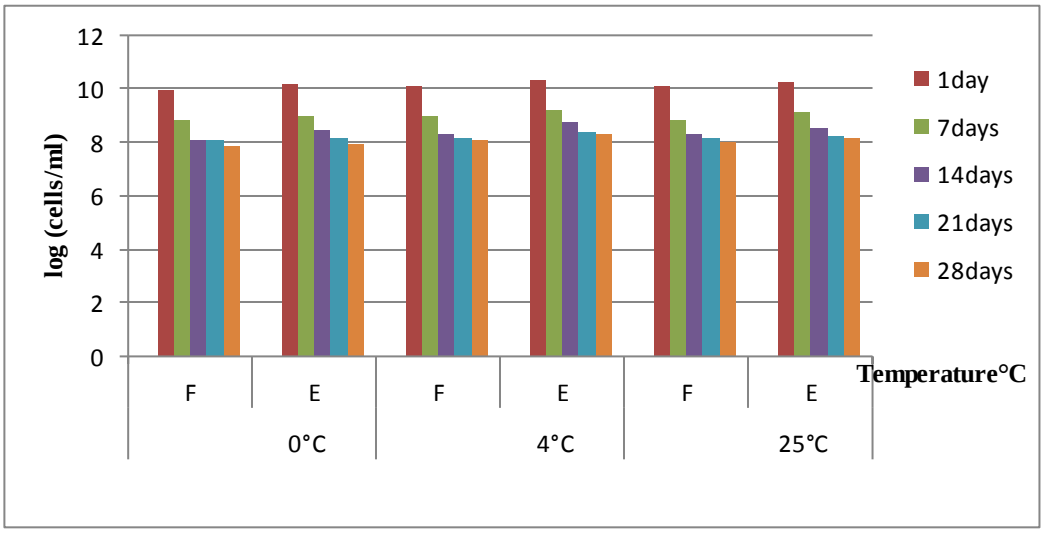

Figure III.9: Survival of free and encapsulated *Lb. brevis* in alginate coated chitosan during storage at different temperatures.

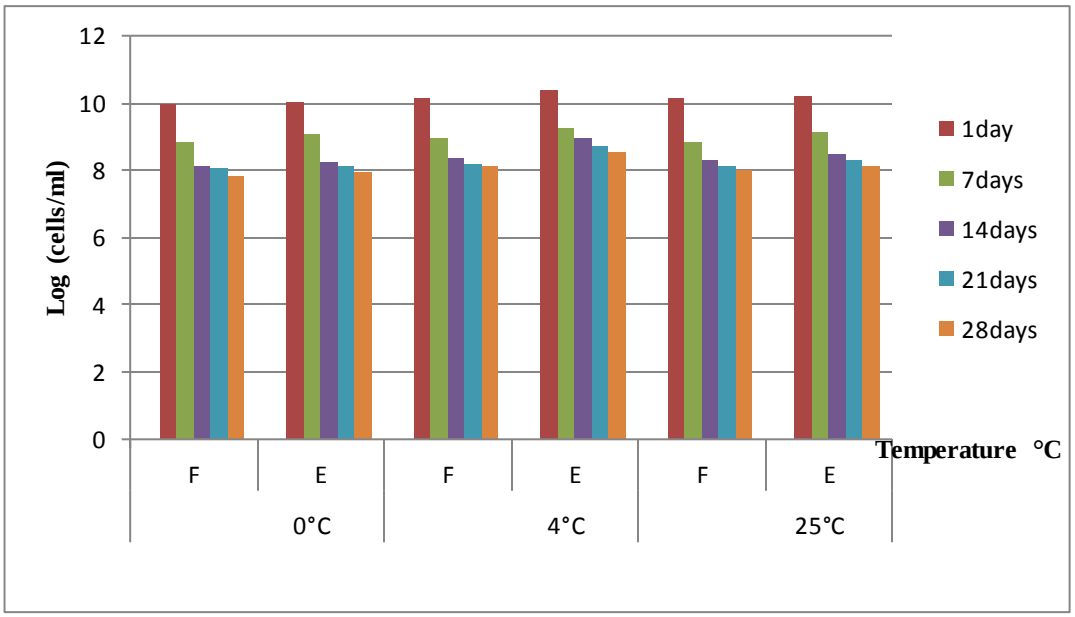

Figure III.10: Survival of free and encapsulated *Lb. brevis* in alginate-glycogen during storage at different temperatures.

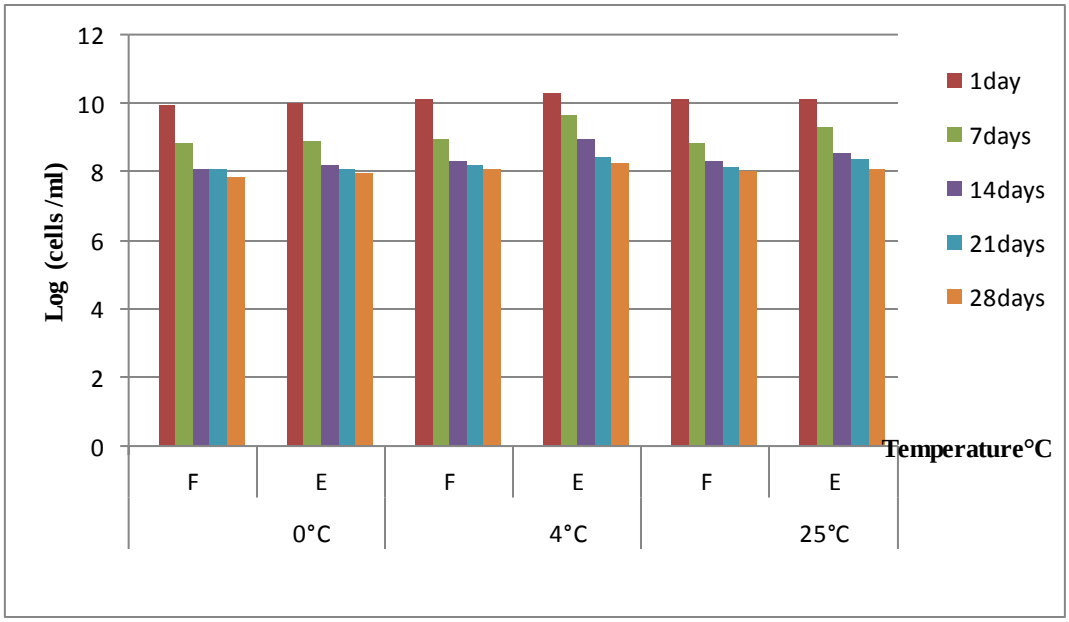

Figure III.11: Survival of free and encapsulated *Lb. brevis* in k-carrageenan during storage at different temperatures.

- ***Lactobacillus plantarum***

Lb. plantarum showed a decrease in number for both F and E cells during 28 days of storage at refrigeration, freezing, and ambient temperature with all polymers (**figure III.12**, **III.13**, **III.14** and

III.15), but the chitosan had the highest number of cells compared with alginate, glycogen and K-carrageenan.

Compared to free cells, the encapsulation process gave a small decrease in the number of bacteria in all polymers and at different storage temperatures, because after 28 days, all encapsulated bacteria demonstrated high viability relative to free cells.

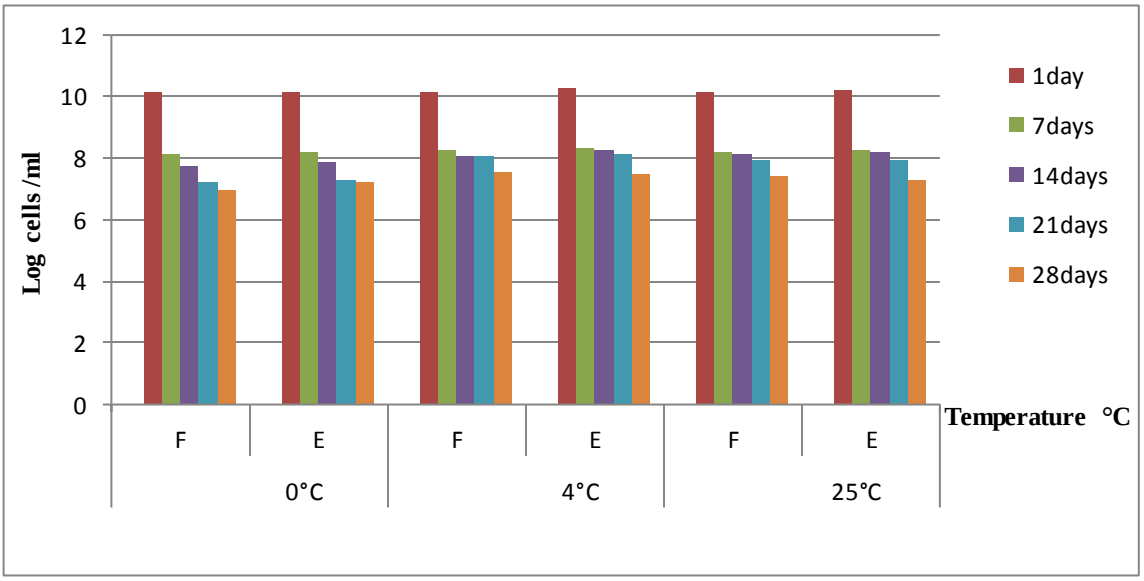

Figure III.12: Survival of free and encapsulated *Lb. plantarum* in alginate during storage at different temperatures.

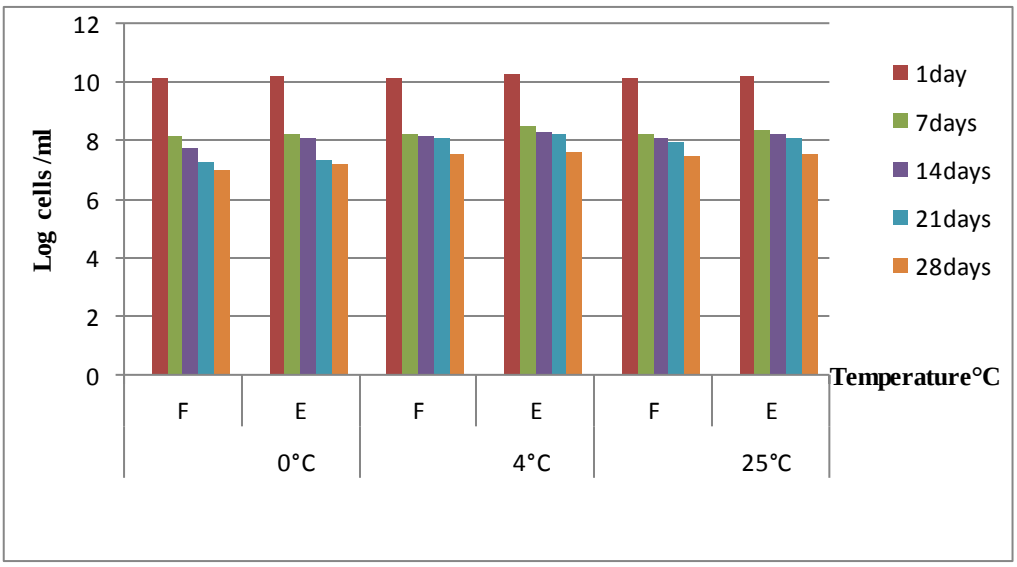

Figure III.13: Survival of free and encapsulated *Lb. plantarum* in alginate coated chitosan during storage at different temperatures.

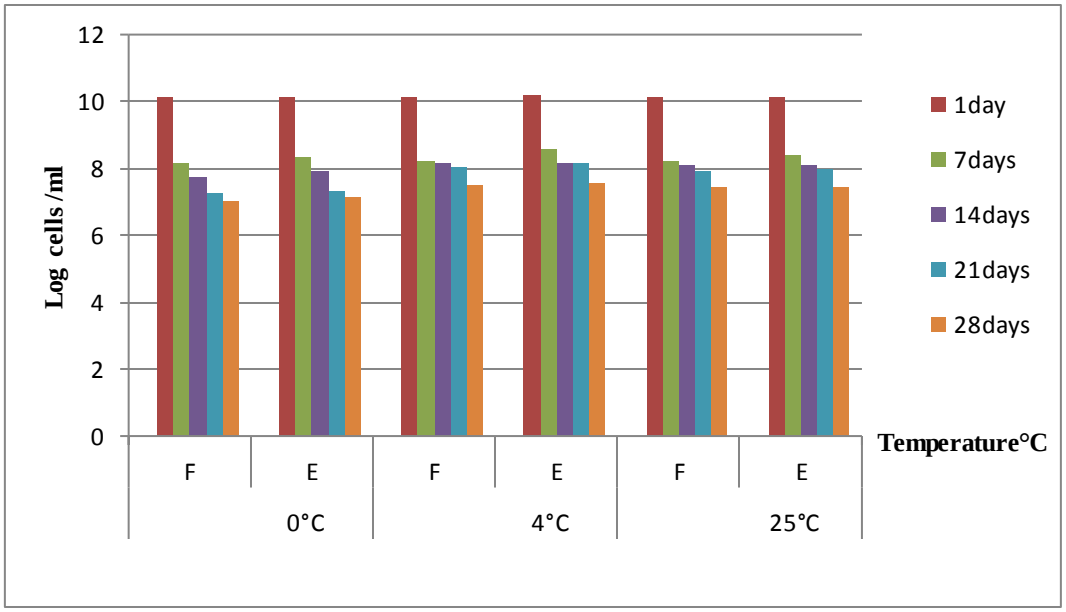

Figure III.14: Survival of free and encapsulated *L. plantarum* in alginate-glycogen during storage at different temperatures.

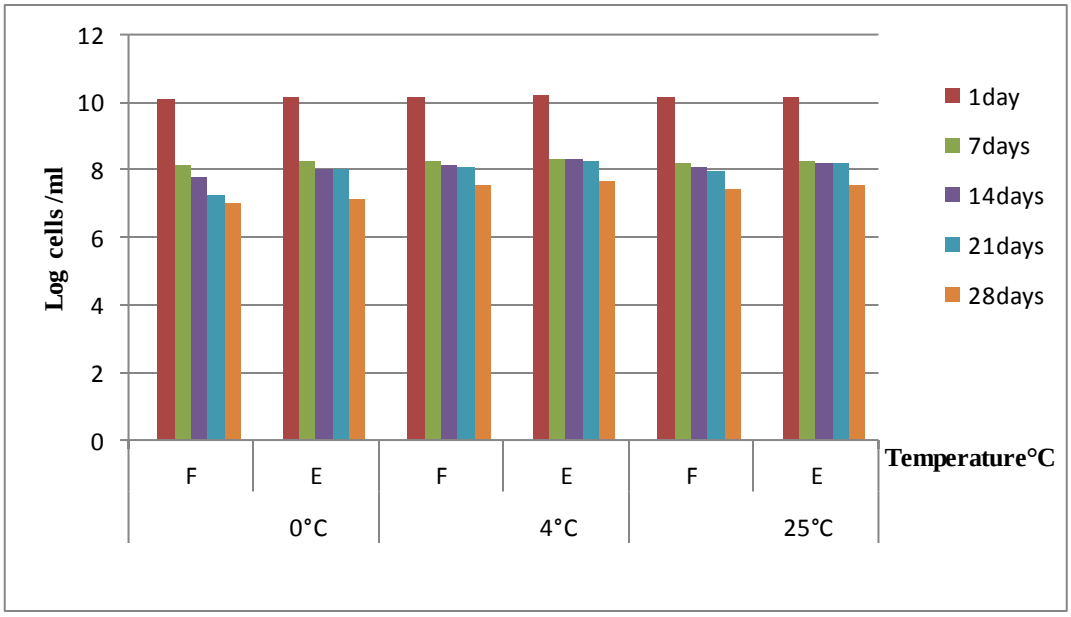

Figure III.15: Survival of free and encapsulated *Lb. plantarum* k-carrageenan during storage at different temperatures.

Ouled Haddar *et al.*, 2013 demonstrated that free cells of ***Lb. curvatus*** are less resistant to storage at 4°C (66% were viable after two weeks of incubation) comparing with encapsulated form, where survival after two weeks remains relatively high (86% survived after two weeks).
According to the above **figures**, there was a small loss in number associated with chitosan coating. This shows that chitosan coating improved the protective effect of alginate microcapsules, whereas the bacteria encapsulated in the alginate-only showed the least protection compared to chitosan, alginate-glycogen and K-carrageenan polymers. According to (**Krasaekoopt *et al.*, 2006; Brinques *et al.*, 2011; Yeung *et al.*, 2016**) this, improved the stability of the beads during storage and could reduce the loss of cells to the medium and positively affect viability.

Moreover, k-carrageenan system showed good stability over the course of 2 weeks but a decrease in number was observed after this time. Treatment with chitosan showed better viability under refrigeration.

In another study of **Fareez *et al.*, 2015** it was indicated that the survival of free and microencapsulated ***Lb. plantarum*** LAB12 after 4 weeks of storage at 4°C was displayed a gradual reduction of the initial counts ranging from 12 to 10 (log CFU/g). While encapsulated cells in alginate, alginate–chitosan, was displayed a cell loss of 18% and 14%, respectively.
Brinques *et al.*, 2011 was founded that the reduction of the number of viable cells of ***Lb. plantarum*** BL011 during 38 days of refrigerated storage at 4°C, chitosan coating system has a reduction of 2.17 log N_0/N whereas alginate system reduction was 4.35 log N_0/N.

Lee *et al.*, 2004 investigated the effect of microparticles of alginate coated with three types of chitosan of different molecular weight on the survival of ***Lb. bulgaricus*** KFRI 673 during the storage at 4 and 22ºC. It was concluded that microencapsulation with alginate and chitosan offers a better coating and a more effective means of viable bacteria, besides of maintaining survival during the refrigerated storage, especially with chitosan of high molecular weight.

Koo *et al.*, 2001 found that the viability of alginate encapsulated cells and alginate-chitosan encapsulated cells decrease slightly after 14 days of storage and reached to $2.2.10^8$ CFU/ml and $7.1.10^9$ CFU/ml, respectively.

Other important observation is that the storage of encapsulated cells at 4°C presents the highest stability and improves the viable cell count. These results are in agreement with the results of **Kim *et al.*, (1988)** which demonstrated that the number of free and encapsulated ***Lb. plantarum*** in sodium alginate was decreased at various storage temperatures; at 4°C the number was 1.6×10^{10} CFU /g during 15 days whereas at 25°C the number of encapsulated ***Lb. plantarum*** was 5.5×10^9 CFU /g.

Koo *et al.*, 2001 indicated that the viable counts of encapsulated ***Lb. casei*** remained stable through the storage periods at 4°C. At 22°C, the viability of encapsulated cells decreased to 2 log-cycle after 7days and continued to decline until the end of storage as 4 log-cycle.

Encapsulated bacteria in alginate-glycogen (prebiotic) presents a good stability at 4°C compared with the non-encapsulated cells and compared to alginate system. These results are in agreement with the studies of **Sultana *et al.*, 2000** who studied the potential for using the prebiotic resistant starch with alginate during encapsulation since it enhances the survival of the probiotic bacteria. They indicated that the addition of starch at 2% concentration to alginate resulted in the recovery of a high number of ***Lb. casei***, which increased from 4.10^8 CFU/ml (0% starch) to $3.1 .10^{11}$ CFU/ml (2% starch). Also**, Martin, 2013** examined the viability of ***Lb. fermentum*** CECT5716, microencapsulated in sodium alginate and sodium alginate combined with starch. The results showed that there was a 3.0 log reduction in the microcapsules of alginate and of only 0.3 log in formulas with alginate and starch.

In fact, the combination of alginate with prebiotics offers an enhanced protection for probiotics in food systems due to the symbiotic relationship. This can be explained by the fact that prebiotics form small aggregates that contribute to a better protection of bacterial cells **(Etchepare *et al.*, 2015)**.

It may also be noted that ***Lb. casei, Lb. plantarum,*** and ***Lb. brevis*** have a different response to the storage conditions. As expected, there was a decrease in the viability of encapsulated cells evaluated, but the rate of the decrease was strain dependent. like the results showed above, ***Lb. brevis*** had the higher survival with all polymers compared with ***Lb. casei*** and ***Lb. plantarum.***

Krasaekoopt *et al.*, (2003) commented that special treatments, such as coating of beads, are used to improve their properties. In these cases, the cross linking with cationic polymers, coating with other polymers, mixed with starch and incorporation of additives could improve the stability of the beads.

III.4. Effect of storage in the pineapple juice on the viability of encapsulated cells

The number of viable cells of encapsulated ***Lb. plantarum***, ***Lb. casei*** and ***Lb. brevis*** stored in pineapple juice is represented in **figures III.16**, **III.17** and **III.18**.

As shown in **figure III.16**, it is clear that encapsulated cells with k- carrageenan showed the highest number of cells after the first day (10.38 (log cells/ml)) this number decrease considerably until the 28 days (8.17 (log cell/ml)), followed by alginate-chitosan encapsulated cells number which is ranged from 9.8 to 8.05 log cells/ml. Encapsulated cells with alginate decrease from 9.5

(log cells/ml) to (8 log cells/ml) and the other with alginate-glycogen from 9.4 (log cells/ml) to 8.02 (log cells/ml).

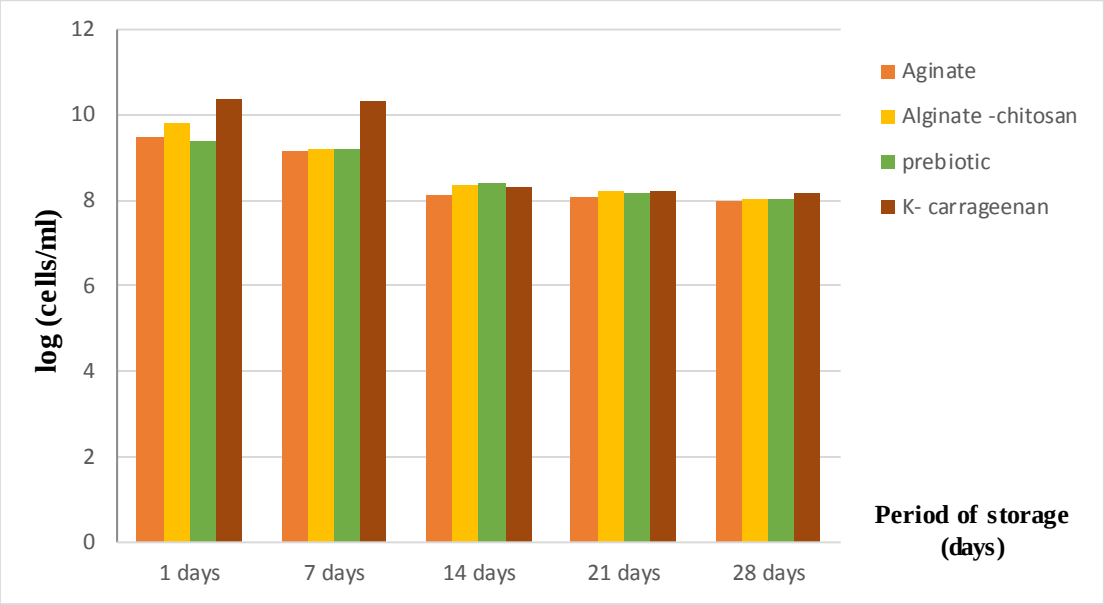

Figure III.16: Number of cells of the encapsulated *Lb. casei* (KBM2) stored in pineapple juice.

As appeared in **figure III.17**, the number of encapsulated cells of each polymer decreased during all the 4 weeks till the 28 days. A quick decrease in the number occurred from the day 1 to the day 8 with alginate (from 10.07 to 8.9 (log cells/ml)), alginate-chitosan (from 10.14 to 8 (log cells/ml)), alginate-glycogen (from 9.88 to 8.07 log cell/ml)) and k-carrageenan (from 10 to 8.14 log cell/ml)), then continue to decrease slowly until the 28 days.

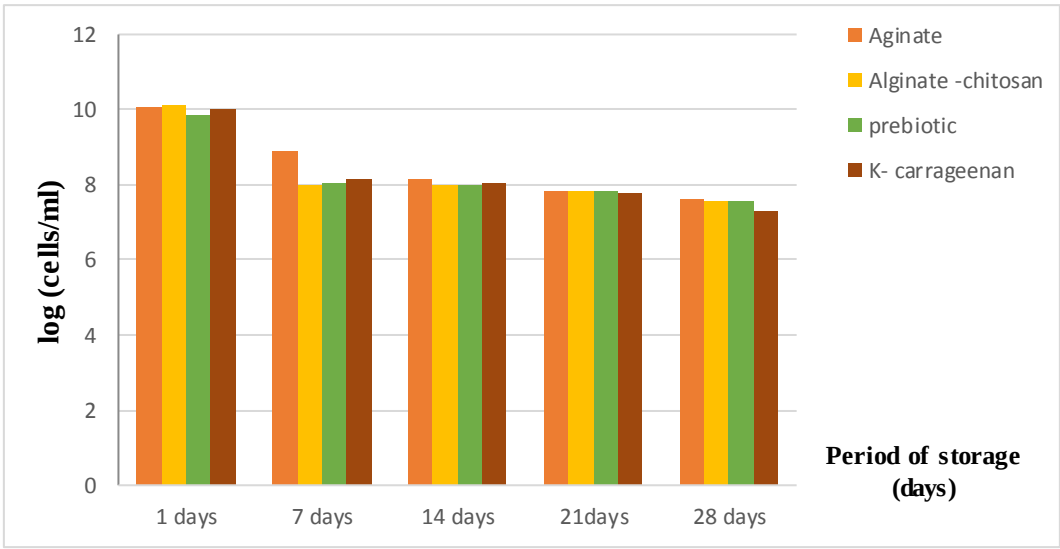

Figure III.17: Number of cells of the encapsulated *Lb. plantarum* (B10) stored in pineapple juice.

The number of encapsulated cells decreased during storage for all polymers as it's represented in **figure III.18,** k-carrageenan has the highest number of cells in the first and the day 7 which reached 10.51 (log cell/ml), 9.8 (log cells/ml) successively and continued to decrease until the 28 days followed by alginate-chitosan polymer which had values near to carrageenan, for the other polymers, the number decreased too.

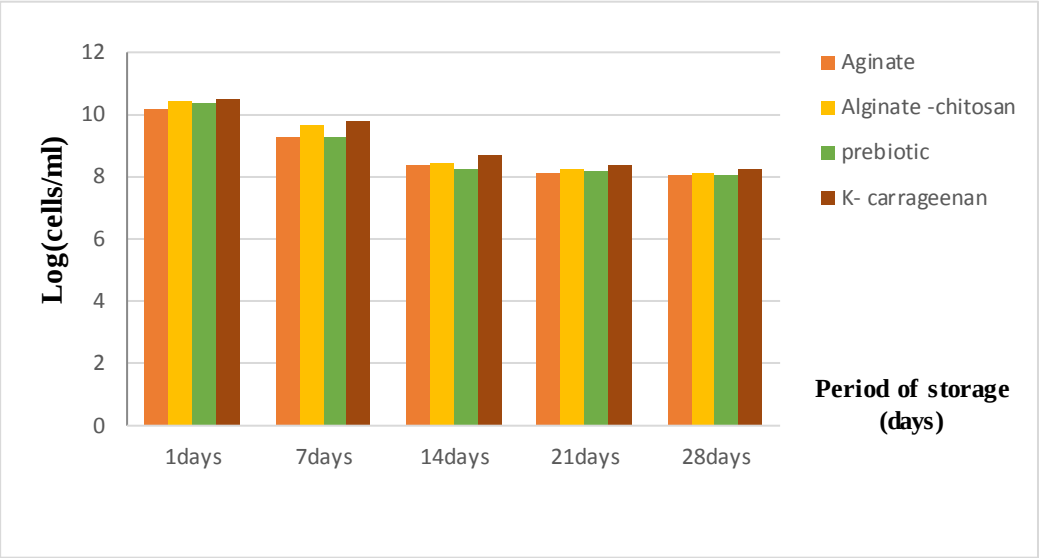

Figure III.18. Number of cells of the encapsulated *Lb. brevis* stored in pineapple juice.

Many factors can influence probiotic survival in fruit juices like species/strain used, the method of preparation of the cultures the composition of the product, the storage temperature and the presence of dietary fibre in the product, the oxygen levels and the type of container **(Nualkaekul and Charalampopoulos, 2011)**.

In order to improve the survival of probiotic strains ***Lb. plantarum***, ***Lb. casei*** *and* ***Lb. brevis*** during storage in pineapple juice, encapsulation in a variety of polymers was studied. In general, the number of encapsulated cells for the three strains decrease during the period of storage in juice but they stay viable at the appropriate number at the end of storage. This result similar to the study of **Ding and shah, 2009** where encapsulated probiotic bacteria protected from the acidic environment of the orange juice did not lose their viability rapidly, which explain the enhancement of viability by a more favorable anaerobic environment for the sensitive probiotic bacteria, as well as a physical barrier from the harsh acidic conditions of the fruit juice.

Our results ensure the data obtained with **Nualkaekul et *al*; 2013** which found that encapsulation with alginate or pectin improve cell survival in the case of ***Lb. plantarum*** in the pomegranate juice.

For ***Lb. plantarum***, alginate remained the suitable polymer that retain the most number of cells, contrary to ***Lb. casei*** and ***Lb. brevis*** where k-carrageenan was the most effective encapsulation material which allows us to conclude that the type of polymer affect the cell viability.

Encapsulation with alginate-chitosan showed the best result with ***Lb. casei*** and ***Lb. brevis*** strain compared to encapsulated cells using alginate only, which demonstrated that the coating material improved the protection offered by the core material on the cells during storage of the cell loaded beads in fruit juices **(Nualkaekul *et al*., 2013)**.

The increased protection by chitosan coating was initially considered to be associated with the increase in the pH of the juice in the case of the coated beads. This increase were most likely due to the fact that chitosan is soluble in various organic acids (**Nualkaekul *et al*., 2012)**. In addition, the combination of alginate with prebiotics offers an enhanced protection for probiotics in fruit juice (**Etchepare *et al*., 2015).**

For all strains, a decrease in the number of encapsulated cells occurred during the period of storage and this is related to many factors like pH and content of the juice in phenolic compounds which have been suggested to have antimicrobial activities against a variety of microorganisms including lactobacilli **(Nualkaekul *et al*., 2013).**

III.5. Effect of storage under simulated gastrointestinal conditions on the viability of free and encapsulated cells

- ***Lactobacillus casei* (KBM2) subjected to gastric and intestinal fluid.**

As it shown in **figure III.19,** the number of F cells of ***Lb. casei*** decrease from 10 (log cell/ml) to 3.44 (log cell/ml) when subjected to the SGS and reach 3.38 (log cell/ml) in the SIS. For the E cells, this number increase considerably using different polymers. Alginate-glycogen show the high number in the SGS that attain 9.53 (log cell/ml) than diminished to 8.9 (log cell/ ml) during exposition to SIS.

Also, E cells in alginate-chitosan show a number of 9.49 (log cells/ml) in SGS that decrease to 9.14 (log cells/ml) compared with E cells in alginate (from 9.43 log cells/ml in SGS to 9 log cells/ml in SIS). For E cell in k-carrageenan the number is the same for both conditions.

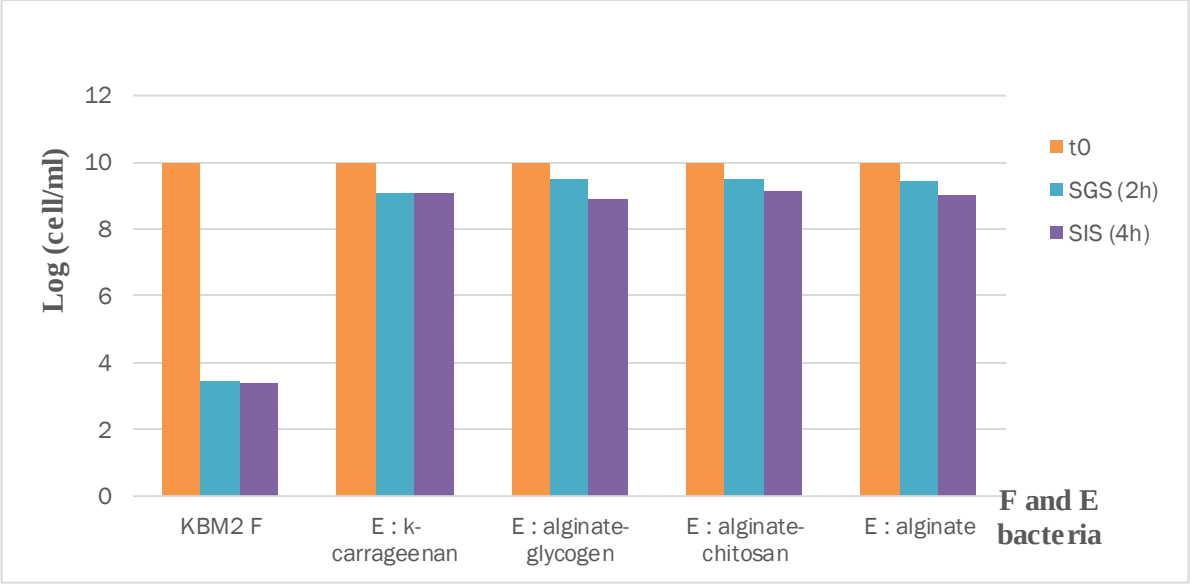

Figure III.19: Number of free and encapsulated cells of *Lb. casei* subjected to SGS and SIS.

- ***Lactobacillus plantarum* (B10) subjected to gastric and intestinal fluid**

In this case (**figure III.20**), number of F cells decreased to 4.63 (log cells/ml) in the SGS and reached 4.36 (log cell/ml) in the SIS. But always the number of E cells keep higher, the great value is 9.74 (log cells/ml) showed by k-carrageenan in the SGS and decrease to 9.14 (log cells/ml) in the SIS.

The E strain with alginate-chitosan resist the SGS to attain 9.64 log cells/ml and diminish to 9.44 (log cell/ml) in the SIS, compared to E cell in alginate where 9.55 (log cells/ml) obtained in SGS decreasing to 9.4 (log cells/m) in the SIS. Whereas, the number of E cells with alginate-glycogen remain stable in SGS and SIS.

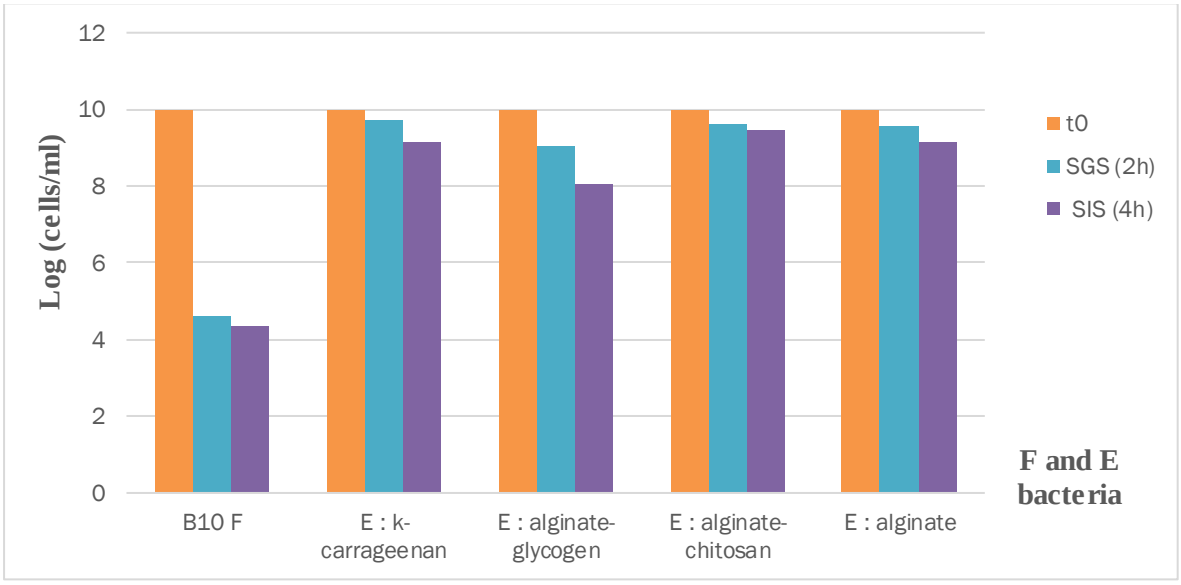

Figure III.20: Number of free and encapsulated cells of *Lb.plantarum* subjected to SGS and SIS.

- ***Lactobacillus brevis* (B1) subjected to gastric and intestinal fluid**

As presented in **figure III.21,** the number of F cells decease in the SGS to reach 3.45 (log cells/ml) and reached 3.15 (log cells/ml) in the SIS. E cells with each polymer show high number of cells in the SGS 5.49 (log cells/ml) for k-carrageenan, 5.44 (log cells/ml) for alginate-glycogen, 5.47 (log cells/ml) for alginate-chitosan and 5.17 (log cells/ml) for alginate, these numbers known a rapid diminution in SIS to reach 3.3 (log cells/ml), 3.19 (log cell/ml), 3.25 (log cells/ml) and 3.17 (log cells/ml) for each polymer respectively.

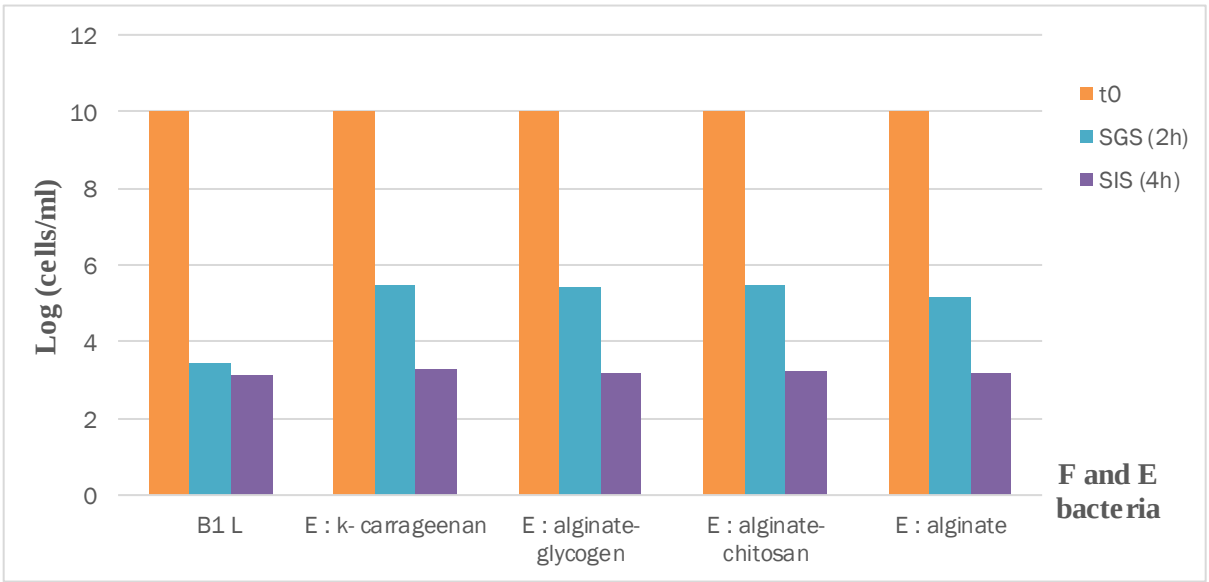

Figure III.21: Number of free and encapsulated cells of *Lb brevis* subjected to SGS and SIS.

Following consumption, microorganisms should survive a transit through the gastric environment and reach the colon in quantities large enough to facilitate colonization. The harsh conditions of the gut, including the acidic nature of the stomach and the presence of bile in the intestine **(Chun *et al*., 2014).** Microencapsulated cells survived better than free cells after sequential incubation in simulated gastric and intestinal juices **(Krasaekoopt *et al*., 2004).**

The F and E cells of each strain showed different responses to the gastric and intestinal conditions. We found that ***Lb. casei*** is the more resistant to the SGS when it's encapsulated with alginate-glycogen and alginate. However, encapsulated ***Lb. plantarum*** with k-carrageenan and alginate-chitosan is the most resistant, and ***Lb. brevis*** remain the lower resistant for each polymer. Which explain that resistance to the gastrointestinal condition depends to the used polymer for encapsulation and to the bacteria strain. Free cells of ***Lb. plantarum*** are more resistance compared with the other strains, show a slight decrease when exposed to SIS.

For all strains, as shown in figures there are a slight difference, always we found that alginate beads coated with chitosan provide more protective effect from harsh gastrointestinal condition compared to the uncoated beads, which explain that chitosan play an additional protective and stabilizing effect.

Our results are similar to those of **Koo *et al*. 2001** and **Chávarri *et al*. (2010)** reported that ***Lb. casei*** and ***Lb. gasseri*** microencapsulated in chitosan-coated with alginate beads had higher viability than microcapsules without chitosan coating in bile salt solution. **Chavarri *et al*. (2010)** found that coating alginate beads with chitosan develops chitosan with alginate complex. This complex decreases the porosity of alginate beads, reduces the leak of the encapsulated probiotic, and shows stability at low pH.

Another study conducted by **Kanmani *et al*. (2011)** showed no released cells of *Enterococcus faecium* MC13 encapsulated into alginate-chitosan capsules in SGS for 144 hours. In contrast, encapsulation into alginate without chitosan failed to protect probiotic cells such as ***Lb. plantarum, Lb. acidophilus, Lb. rhamnosus***, and bifidobacteria in SGS **(Shori, 2017).**

In our study, ***Lb. casei*** encapsulated with glycogen combined with alginate give high resistance to the gastrointestinal condition, like another study where combination of calcium alginate with prebiotics such as inulin not only improve the viability of probiotics but facilitates the formation of an integrated structures of capsules **(Khosravi Zanjani *et al*., 2014).**

Conclusion

Microencapsulation is the main solution to protect probiotics in food and human gut. Our work interests to demonstrate the effect of extrusion method on probiotic viability during storage under different temperatures (0°C, 4°C, 25°C) and storage in pineapple juice, also for their gastrointestinal resistance. Three strains were used: ***L. plantarum***, ***L. casei*** and ***L. brevi*** which were encapsulated with four different polymers: alginate, alginate-chitosan, glycogen and k-carrageenan. The results demonstrated that:

- Microencapsulation using the four polymers enhance the viability of these strains during storage under 0°C, 4°C and 25°C, but the highest viability was recorded at 4°C.
- ***L. casei*** gave the best viability with k-carrageenan, ***L. brevis*** with glycogen and ***L. plantarum*** with chitosan at 4°C.
- Comparing the strains and matrices, ***L. brevis*** showed the highest cells number with all encapsulation polymers during storage at 4°C.
- All encapsulated cells with all polymers exhibited a good viability after their storage at pineapple beverage during the period of storage.
- A good improvement was obtained in viability of all strains and polymers when exposed to gastrointestinal conditions.

We conclude that microencapsulation enhanced the viability of the three strains when exposed to the GIC, storage in juice and at different temperatures.

Future researches are recommended to test the viability of the same strains with other polymers and in other beverages and food matrices.

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Appendices

1- MRS broth :

Table 1 : The composition of the MRS (Man Rogosa Sharp) for 1L

Components	**Quantity**
Glucose	20g
Yeast extract	8g
Meat extract	4g
Sodium acetate	5g
Bipotassic phosphate	2g
Ammonium citrate	2g
Magnesium sulfate	0.2g
Manganese sulfate	0.05g
Tween 80	1 ml
Distilled water	1000ml

2- MRS agar :

MRS broth with 15g of agar.

3- Calcium chloride $CaCl_2$ 0.5M for 1L : 55.49g de $CaCl_2$ in 1L of distilled water.

4- Sodium citrate 5%: 5g of Sodium citrate dissolved in 100ml of distilled water.

5- Physiologic water: 9g of NaCl dissolved in 1000ml of distilled water.

6- Sodium alginate 2%: 2g of sodium alginate powder in 100 ml of distilled water.

7- Chitosan 0.8%: 0.8g of chitosan powder in 100 ml of distilled water.

8- Alginate (1%) – k- carrageenan (1%) polymer: 1g of sodium alginate in 100 ml of distilled water and 1g of k-carrageenan in 100 ml of distilled water.

9- Alginate (2%) – glycogen (1%) – glycerol (2.5%) polymer: 2g of sodium alginate, 1g of glycogen and 2.5 ml of glycerol each one in 100 ml of distilled water.

10- Juice composition

Composition of pineapple juice of N'gaous, UHT:

Water, sugar, concentrate of pineapple, concentrate of apple, stabilizing:(pectin, carob gum) (1.14g/l), colorant, aroma, carotene, acidity regulator, citric acid, antioxidant, ascorbic acid (vitamin C).

11- Gastric solution

Table 2: Composition of the gastric solution.

Components	**Quantity**
NaCl	5g/l
KCl	2.2g/l
$NaHCo_3$	1.2g/l
$CaCl_2$	0.22g/l

12- Intestinal solution:

Table 3: Composition of the intestinal solution.

Components	**Quantity**
$NaHCo_3$	6.4g/l
KCl	0.23g/l
NaCl	1.28g/l

13- Buffer solution

Composition of phosphate buffer for 250ml:

K_2HPO_4 solution: 69.6g of K_2HPO_4 dissolved in 200ml of distilled water.

KH_2PO_4 solution: 13.5g dissolved in 50ml of distilled water.

Realised by

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Effect of encapsulation in sodium alginate, k-carrageenan and chitosan on the viability of some Lactic Acid Bacteria during storage, under juice and gastrointestinal conditions.

Abstract

The aim of this work was to evaluate the effect of storage on the viability of free and encapsulated lactic acid bacteria. Encapsulated cells of *L. brevis*, *L. casei*, and *L. plantarum* were prepared by extrusion in 2% sodium alginate, alginate-chitosan, k-carrageenan and alginate-glycogen gels.Storage temperatures were25°C, 4°C and freezing for1, 7, 14,21and 28 days. Commercial juice(pineapple) have been also used to store beads of different gels at 4°C.Moreover, encapsulated cells were exposed to gastrointestinal conditions.

results showed that encapsulated cells survived better than free ones in all conditions and with all gels used. in addition *L. casei* gave the best viability with k-carrageenan, *L. brevis* with alginate-glycogen and *L. plantarum* with chitosan at 4°C.

Key words: encapsulated cells, gastrointestinal conditions, storage.

Résumé

L'objectif de ce travail était d'évaluer l'effet de la conservation sur la viabilité des bactéries lactiques libres et encapsulées. l'encapsulation a été réalisée par l'extrusion dans les gels d'alginate, alginate-chitosane, k-carrageenan et alginate-glycogène à 2%. Les températures utilisées de conservation étaient 4°C, 25°C et la 0°C pendant 1, 7,14, 21 et 28 jours. le jus d'ananas commercial a été utilisé pour conserver les billes a 4°C. en, plus, les billes ont etéexposé aux conditions gastro-intestinales .

les résultats obtenues indiquent que toutes les cellules encapsulées résistaient les conditions citées avec tous les polymères et *L. casei* avait la meilleure viabilité avec k-carrageenan, *L. brevis* avec l'alginate- glycogene et *L. plantarum* avec chitosan at 4°C.

Mots clés: Bactéries encapsulées, conditions gastro-intestinales, conservation.

الملخص:

الهدف من هذه الدراسة هو تقييم تأثير الحفظ على حيوية البكتيريا اللبنية الحرة والمغلفة ، و قد تمت الكبسلة باستعمال هلام الالجينات، الالجينات و الكيتوزان الكراجينان والالجينات–الجليكوجان 2.% درجات الحرارة المستعملة للتخزين هي ،°4م،°0 م،25 م لمدة1 7،14،21و28 يوم. وقد تم استخدام عصير الاناناس التجاري لحفظ الخلايا الحرة والمغلفة في درجة حرارة°4 .م اضافة الى ذلك، تم تعريضها لظروف الجهاز الهضمي.

النتائج المتوصل اليها بينت ان السلالات المغلفة قاومت جميع الظروف مع جميع انواع الهلام و ابدت *L. casei* مقاومة افضل مع الكاراجنين *L. brevis* مع الجليكوجان و *L. plantarum* مع الكيتوزان

الكلمات المفتاحية: بكتيريا مغلفة ، ظروف الجهاز الهضمي ، الحفظ.