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Realized by **Samiya AMIRA**

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Effect of microencapsulation on the viability of probiotic bacteria under storage and simulated gastrointestinal conditions

Under the Supervision of: **Pr. Mohammed SIFOUR**

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List of abbreviations

ADH: arginine dihydrolase.

CAP: cellulose acetate phtalate.

CFU: colony forming units.

GIC: gastrointestinal conditions.

GSJ: gastric simulated juice.

ISJ: intestinal simulated juice.

LAB: lactic acid bacteria.

MRS: Man Rogosa and Sharp medium.

^{o}D: Dornic degree.

PBS: phosphate buffer saline.

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Introduction

Recently, it becomes possible that a food serves as medication, fights diseases and contributes to good health. Hence new concepts with specific terms appeared, *i.e.* functional foods or super foods. The same meaning was previously proposed by Hippocrates when he said "Let food be the medicine, and let medicine be the food" **(Elmalikis *et al.*, 2019)**. In fact, the denomination of functional foods is related to components they contain, such as natural or added specific minerals, vitamins, fatty acids, dietary fibers and biologically active substances such as phytochemicals, antioxidants, and probiotics and also related to the consequences they have for health enhancement and decrease of diseases risk **(Koutelidakis and Dimou, 2016; Chaudhary, 2019)**.

Lactic acid bacteria (LAB), is an important group of probiotics, well known by their health effects. In fact, they contribute to normal intestinal microflora balance, reduce gastrointestinal and urogenital pathogens number, lower serum cholesterol level and blood pressure and in some cases prevent some cancers (**Bron *et al.*, 2012; Khani *et al.*, 2012; Amine *et al.*, 2014)**. All these properties render lactic acid bacteria successful candidates to be used for functional foods. They are usually added as a concentrated culture to a beverage (fruit juice for example), as a freeze-dried dietary supplement formulated in solid dosage forms (powder, capsules, tablets) or inoculated in prebiotic fibers or in milk based foods **(González-Ferrero *et al.*, 2018)**.

According to **Lee and Salminen (1995)**, the daily recommended intake of probiotics to be health effective is at least 10^6-10^9 living cells, which means that a final processed functional food including stored foods should contain at least this number. Besides, we have to take into consideration the harsh gastrointestinal environment such as acidity of stomach and destructive enzymatic machinery (pepsin), and the intestinal conditions with their basic environment containing pancreatin and bile salts through which probiotics pass to exert their therapeutic properties. At this regard, the challenge of micro-encapsualation appeared as a real potential technology to sustain probiotics viability from their processing (biomass production, lyophilisation, storage, application in food) up to their passage through gastrointestinal tract **(Chavarri *et al.*, 2010; Chen *et al.*, 2017)**.

Microencapsulation of probiotics is a way of covering living cells in order to protect them and release them under appropriate conditions. It uses a variety of materials or polymers, also known as matrices, which are commonly introduced as food additives, and are all originating from nature, like alginate and carrageenan from algae, starch, gum Arabic, soy and pea

protein from plants, gellan and xanthan from bacteria and milk, gelatin and whey protein from animal origin. These polymers were reported to be biologically compatible and safe for both bacteria and consumer. They are applied with a multitude of techniques such as direct gelation, emulsification or complex coacervation. Characteristics of the final obtained capsules such as morphology, texture, size and porosity and also functional properties are material and technology dependant **(Dong *et al.*, 2013; Bosnea *et al.*, 2014; Wang *et al.*, 2014; Eratte *et al.*, 2015)**. Vesale Pharma (probiotic food supplements producer), Lallemand which developed Probiocap® (a probiotic microencapsulation technology) and the French Capsulae (research and development company that offers customized solutions in the field of microencapsulation) are some examples of research foundations interested in enhancement of microcapsules and selling, in Europe **(Deprisco and Maueriello, 2016)**.

Klila "cheese" is one of the most common Algerian traditional fermented dairy products, consumed in a fresh or dried form. It is a very nutritional product due to its components (high protein and calcium and low fat content). It is important to note that besides its chemical composition, it harbors a multitude of microorganisms considered as indigenous microorganisms (lactic acid bacteria) which influence the nutritional, organoleptic and safety of the final products **(Boubekri and Otha, 1996; Mennane *et al.*, 2007; Leksir *et al.*, 2019).**

We aim via this work to isolate and identify lactic acid bacteria from an Algerian traditional fermented dairy products "Klila". The obtained isolates were further characterized for their probiotic and technological aptitudes in order to select the more potent. The aim is also extended to encapsulate the selected bacteria in a variety of polymers and to study the effect of cold storage and the simulated gastrointestinal conditions on the viability of the encapsulated bacteria. Finally, the encapsulated bacteria were introduced in food matrices (pineapple and strawberry beverages) and their viability under storage in cold conditions was studied.

Literature review

I.1. Lactic acid bacteria and probiotics

I.1.1. Probiotics

Probiotics is a Greek word meaning for life, however, the accepted definition is that given by **FAO and WHO (2002)**, "probiotics are living microorganisms which, when administered in adequate quantities, confer benefits to the host health". The amount of probiotics that food should contain to be efficient and to be benefic is at least 10^6 CFU/ml or a daily intake between 10^8 and 10^{11} CFU/day **(Uriot *et al.*, 2017)**. Moreover, this number must remain stable, throughout the storage time of the product **(Saad *et al.*, 2013)**. In general, there is a recent agreement that the population requirement of probiotic cultures to exert health beneficial effects to the consumer can vary depending on the strain and the expected beneficial effect **(Sireswar *et al.*, 2017)**.

Probiotics include a variety of microorganisms, however, lactic acid bacteria (LAB) are the most used in foods principally *Lactobacillus* spp. and *Bifidobacterium* spp., *Enterococcus faecalis*, *Enterococcus faecium*, *Lactococcus* and *Sporolactobacillus inulinus*, and also non-lactic acid bacteria including *Bacillus cereus*, *Saccharomyces cerevisiae* and *Saccharomyces boulardii*, *Escherichia coli* Nissle 1917 and *Propionibacterium freudenreichii* are also considered as probiotics **(Homayouni, 2014)**.

I.1.2. Lactic acid bacteria

Lactic acid bacteria are microorganisms sharing the following features: they are Gram-positive, non-spore forming, cocci or rods, catalase-negative, usually non motile, catalase negative, devoid of cytochromes and they are also characterized by their tolerance to low pH **(Van Geel-Schuttena *et al.*, 1998; Kaban and Kaya, 2008)**. Lactic acid bacteria ferment carbohydrates to obtain energy, using endogenous carbon sources as the final electron acceptor instead of oxygen. They are aerotolerant, and are protected against oxygen by-products such as hydrogen peroxide by peroxidases. Phenotypic methods have been most commonly used for the identification of LAB (physiological and biochemical characteristics), however, molecular characterization becomes an efficient tool for identification, it includes: random amplified polymorphic DNA profiling, 16S rRNA gene sequencing, PCR-based fingerprinting and soluble protein patterns **(Salminen *et al.*, 1998)** and differentiation of species by multiplex PCR assay by using specific recA derived primers **(Torriani *et al.*, 2001)**.

Cellular morphology, temperature growth range, glucose fermentation mode and also sugar use pattern are the characteristics on which classification of LAB is depending on. LAB are currently belonged to the order *Lactobacillales*, class of *Bacilli* and the *Firmicutes* phylum **(Quinto *et al.*, 2014)**. Six families of LAB belonged to the order of *Lactobacillales*, they are *Aerococcaceae, Carnobacteriaceae, Enterococcaceae, Lactobacillaceae, Leuconostocaceae, and Streptococcaceae* **(Sun *et al.*, 2015; Mozzi *et al.*, 2016)**. Each family includes its corresponding genera as shown in **table I.1**.

According to the final products of carbohydrates fermentation, LAB are distinguished into two categories homofermentative and heterofermentative microorganisms. If the final product is only lactic acid, LAB are called homofermentative whereas heterofermentative LAB produce lactic acid, acetic acid or alcohol and carbon dioxide **(Mokoena *et al.*, 2016)**. It is important to mention that Bifidobacteria carbohydtates fermentation end products are also lactate and acetate however the pathway followed is fructose-6-phosphate differs from metabolic pathway of LAB. Moreover, C and G percentage is more than 50 mol%, and are not related to LAB but to Actinobacteria **(Bjorkroth and Koort, 2011)**.

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

Family	Genus
Aerococcaceae	*Abiotrophia, Aerococcus, Dolosicoccus, Eremococcus, Facklamia, Ignavigranum, Globicatella,*
Carnobacteriaceae	*Alkalibacterium, Allofustis, Alloiococcus, Atopobacter, Atopococus, 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*

LAB are highly needed in the following field: fermentation of milk, vegetables, sausages, beverages, and bakery products, furthermore, they are responsible of modification of the composition and flavor of the products. LAB can confer the following benefits: they improve the absorption of nutrients (mainly calcium) in the intestinal tract, also, they modulate the immune system, they exhibit antihypertensive effect, they have antimicrobial activity through

the production of organic acids, carbon dioxide, hydrogen peroxide, ethanol, aromatic compounds and bacteriocins **(Calo-Mata *et al*., 2008)**.

LAB can be isolated from different sources, showing that their habitats are various. For example *Lactococcus* and *Lactobacillus*, *Enterococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcu*s are commonly detected in milk and milk products **(Stiles and Holzapfel, 1997)**. LAB are widely distributed in nature and they could be isolated from soils, water, plants, silages, waste products, oral cavity, gastrointestinal tract and genitalia of animals and humans **(Konig *et al*., 2017)**. Other sources such as traditional fermented foods, traditional fermented drinks, vegetables, and fruit juices are also interesting sources of LAB **(Siddiqee *et al*., 2013; Schoster *et al*., 2014)**.

I.1.3. Screening and selection of probiotics

Probiotics have properties that vary depending on the species or microbial strain. The first criterion is that they must be GRAS (generally recognized as safe), which means being non pathogenic, not allergic and not being able to transfer any antibiotic genes to other bacteria **(Sornplang and Piyadeatsoo, 2016)**. The second important criterion is the resistance to harsh conditions in order to be alive in the target site. In addition to these features, to be considered and used as probiotics, LAB may possess the following criteria: survive, proliferate and colonize their specific locations, have intestinal epithelial adhesion properties, and have the ability to inhibit known pathogens and spoilage organisms **(Daliri and Lee, 2015)**.

The human origin of probiotics is a condition, and despite the diversity of sources of LAB, to use a probiotic strain for the human purpose, it should be isolated from human microflora system which will have the ability to adhere more in the human intestinal cell walls than the others and also must be safe **(Gupta *et al*., 2018)**.

Another condition is the viability in some conditions of stress and food, storage, and till the end of the shelf life of the products; and must survive and colonize while passing through the gastrointestinal tract **(Ali, 2010; Brinti and Shind, 2011; Ravinder *et al*., 2012)**. Adhesion to the intestinal epithelial tissue is a primordial criterion for colonization and thus producing health benefits by competing with the other bacteria, and exerting antagonistic effect against harmful pathogens **(Tuomola *et al*., 2001; Marco *et al*., 2006)**. The adhesion of LAB to the intestinal epithelial tissue is related to a good adherence ability, to flow-rate of the LAB through the gut and to the presence of mucins to trap, protect and lubricate the intestinal

surfaces. Furthermore, adhesion of LAB may involve the binding of LAB to a specific cell surface receptor or it may bind to extracellular matrix such as collagen **(Howard *et al.*, 2000)**.

I.1.4. Health benefits and mechanisms of action of LAB

It is well known that the digestive system of humans at birth is empty from microflora, however, the acquisition of microflora appeared through contact to environment and through food consumption. The use of probiotics is valid whenever the balance of the intestinal microflora is broken, the organism itself becomes unable to return to normal. However, the use of probiotics seems to be efficient since they compete with the other bacteria as shown in **figure I.1**, inhibiting their growth and ultimately leading to the restoration of biological balance **(Sanders, 2008; Sornplang and Piyadeatsoo, 2016)**.

The mechanisms of action of probiotics on the host are complex and some of them are as follow **(Chen *et al.*, 2013; Bakirtzi *et al.*, 2016)**: they serve as supplement to the host microflora and provide protection against various enteric pathogens; they strengthen the gut barrier by competition with pathogenic microbiota for adhesion to the gut; they stimulate and regulate the immune response by initiating the activation of specific genes of localized host cells. They regulate chronic inflammation in intestinal mucosal tissue; this suggests that a direct contact of these probiotics with the various constituents of the intestinal barrier such as endogenous microflora, intestinal mucus and epithelial cells is necessary.

In fact, probiotics affect the immune system by induction of the production of chemokines, cytokines, regulatory T cells, activation of dendritic cells and macrophages and also stimulation of the production of specific antibodies and mucous **(Harzallah and Belhadj, 2013; Ranadheera *et al.*, 2014; Shewale *et al.*, 2014)**. They contribute also in decreasing the duration and preventing intestinal diseases such as Inflammatory Bowel Disease, diarrhea and constipation by colonizing and modulating gut microflora **(Van Geel-Schuttená, 2008; Gupta, 2011)**. It was reported that prevention of diarrhea was related to the consumption of food enriched with probiotic lactic acid bacteria, this food results in the production of lactoferrin, bioactive peptides, flavonoids and many dietary compounds which help in maintaining the gut microflora **(shewale *et al.*, 2014)**, antioxidants and anti-inflammatory, neuropeptides, and polyamines that modulates and also benefits brain health **(Harzallah and Belhadj, 2013)**.

diabetes, obesity and cardiovascular diseases may be also prevented by enhancing gut microbiota, restoring antioxidant system, decreasing insulin resistance and inflammation

(Parvez *et al.*, 2006); in addition, probiotics may prevent cancer, by detoxification of chemical carcinogens, decreasing the release of toxic metabolites, enhancing antioxidant system, modulating immune response to inhibit self-proliferation of cancer **(Kim *et al.*, 2003; Kumar *et al.*, 2010)**; LAB found to be efficient in lactose intolerance by providing β-galactosidase (Lactase) enzyme **(Parvez *et al.*, 2006)**. They have also benefits in lowering the cholesterol level by precipitating cholesterol with free bile salts into bile acids and thereby reducing cholesterol absorption **(Park *et al.*, 2018)**.

Moreover, harmful pathogens maybe also excluded by probiotic LAB and this by different mechanisms such as adhesion to the active site **(Bikila, 2015; Gupta *et al.*, 2018);** and production of different substances with antimicrobial effect, (bacteriocins, lactic acid, acetic acid, propionic acid, alcohol, and diacetyl). These compounds interact with the cell membranes of the pathogens and lower the intracellular pH and thus inhibit them or disrupt their membrane permeability through pores formation **(Aymerich, 2000; Saraniya and Jeevaratnam, 2014)**.

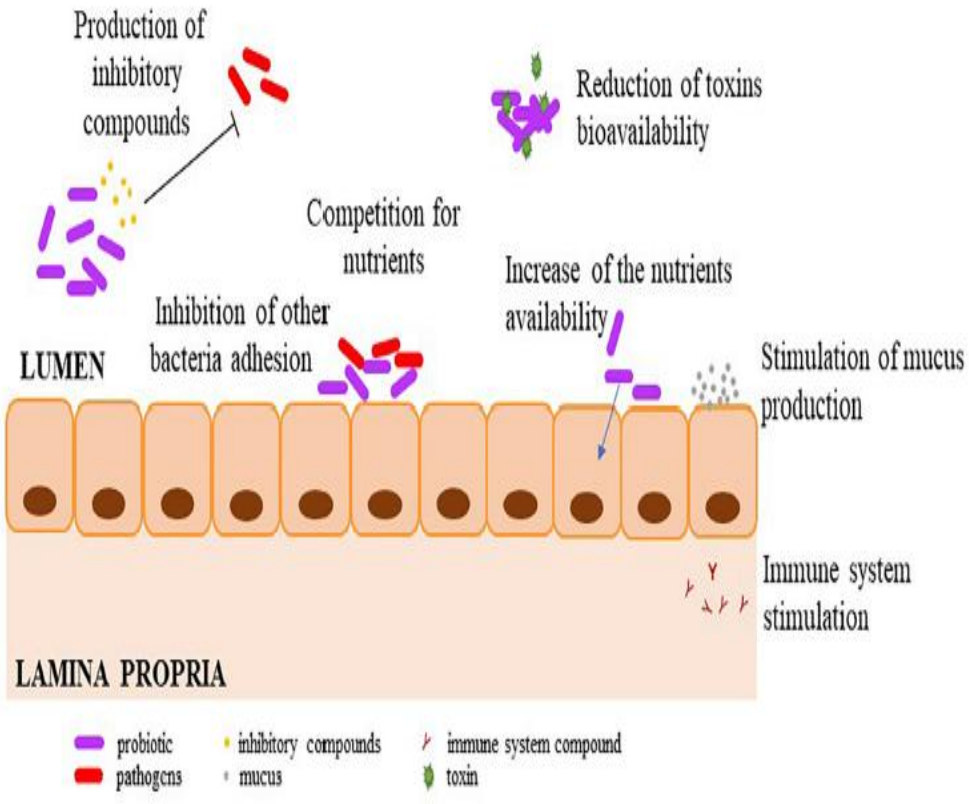


Figure I.1. Mechanisms of pathogen inhibition by the probiotics LAB **(Vieco-saiz *et al.*, 2019)**.

I.2. Klila

Mediterranean countries are characterized by their variety of cheeses produced traditionally. The most popular of them in North Africa are Jben, Lben, Klila and Raib **(Mechai and Kiran, 2008)**. In Algeria, and till now, 10 types of traditional cheeses were characterized, some are consumed fresh while others in a dry form and they are produced throughout the country, from the north to the south and from the east to the west. The well kown ones are Klila and Jben while the less known are Bouhezza, Mechouna and Madeghissa in the east of Algeria (Chaouia region), Takammèrite and Aoules in the south or Igounanes in the middle north (Kabily region). All these cheeses have some common steps, coagulation, draining, salting and for some cheeses also ripening. However, bouhezza seems to be the only ripened traditional cheese (**figure I.2) (Mechai and Kiran, 2008; Leksir *et al.*, 2019)**. Despite this variety in cheeses, industrial ones appeared more popular and consumers still know little about them because their production is generally at a small, local scale.

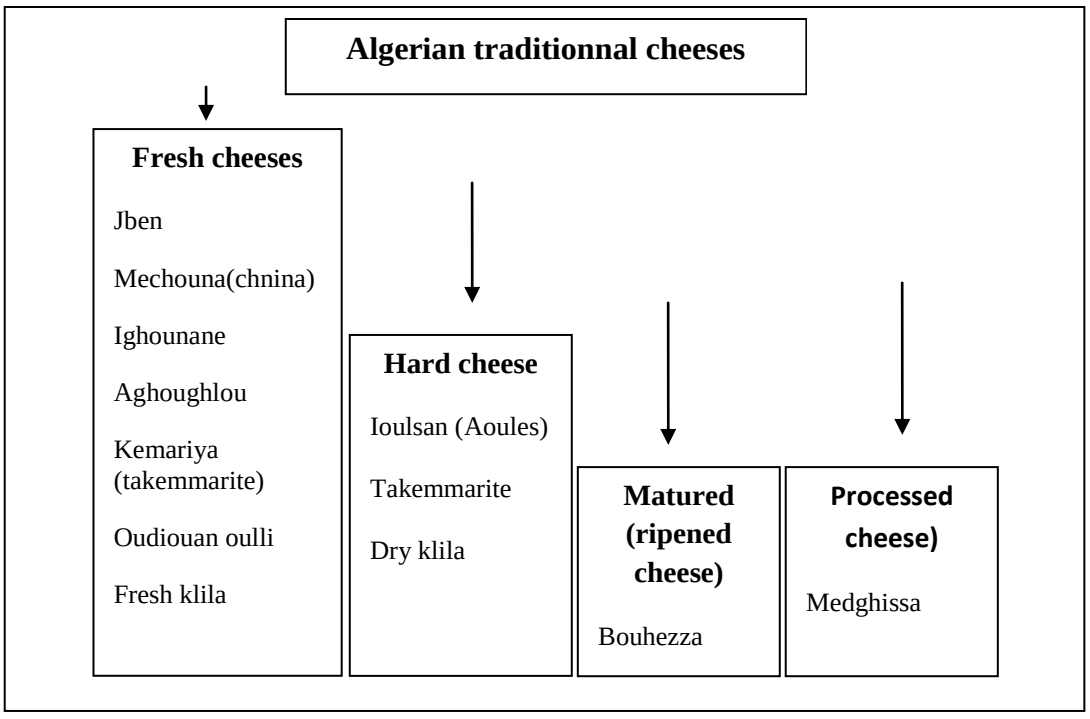


Figure I.2. Algerian traditional cheese **(Leksir *et al.*, 2019)**

The cheese Klila has been consumed by the Algerian people for many centuries, probably from as far back as the Antiquity until now **(Leksir *et al.*, 2019)**. It is one of the most well-known traditional fermented dairy products (cheese in Algeria), it is rich with nutritious compounds of varied flavors, aromas, and textures. This cheese is based on the metabolic

activity of LAB to ferment sugars, especially glucose and galactose, so to produce lactic acid and aroma substances that give typical flavors and tastes to the fermented products.

The Klila cheese is produced in steppe and mountainous areas. It is traditionally made with milk of ewe, goat or cow **(Boubekri and Otha, 1996)**. In fact, dry Klila is characterized by its long period of storage because it contained more than (> 90%) dry matter which leads to its safety from microbial spoilage **(Leksir and Chemmam, 2015)**.

For denomination, different propositions may be given since the right meaning and the right first use still unknown. According to **Leksir and Chemmam (2015),** probably, the origin of the term Klila is Berber meaning ikil a curdled milk, or Tiklitt (the milk that curdles spontaneously), the Arabic meaning may be related to the amount of cheese produced compared to the initial amount of milk (it is few), another denomination appeared in the north east of Algeria "Lagta", the same word used by Berbers but for low-fat cheese made and dried under the sun's rays. Another denomination is "Lemjeben" in other regions which is also a sun-dried cheese **(Leksir *et al.*, 2019)**.

The cheese fermentation, like many traditional fermenting processes, is spontaneous and uncontrolled and so involves several food microorganisms whose type are influenced by the environmental conditions of the area where the cheese is produced. Microorganisms which are responsible for the acid production in cheese making are LAB **(Boubekri and Otha, 1996)**.

Benamara *et al.* (2016) reported that the microbiological and biochemical characterization of Klila prepared from the previously mentioned milks showed the presence of *Lactobacilli* and *Enterococci* where *Lb. plantarum* was the main specie isolated, followed by *Pediococcus pentosaceus, Leuconostoc pseudomesenteroïdes* and *Lb. fermentum*. The *Enterococcus* genus was dominated by *Ec. durans, Ec. faecium* and *Ec. hirae*. Probably, these microbes are representative of the environmental context in which Klila is produced.

Klila is obtained from fermented churned milk called *Lben*. *Lben* is heated, drained and pressed to obtain Klila (**figure I.3**) **(Benamara *et al.*, 2016)**. This popular cheese is still based on a traditional farmhouse production method which contributes to the pleasant sensory attributes and nutritional properties; which explains the increasing consumer demand for

Klila. Unfortunately only few microbiological, biochemical and technological data are available **(Leksir and Chemmam, 2015)**.

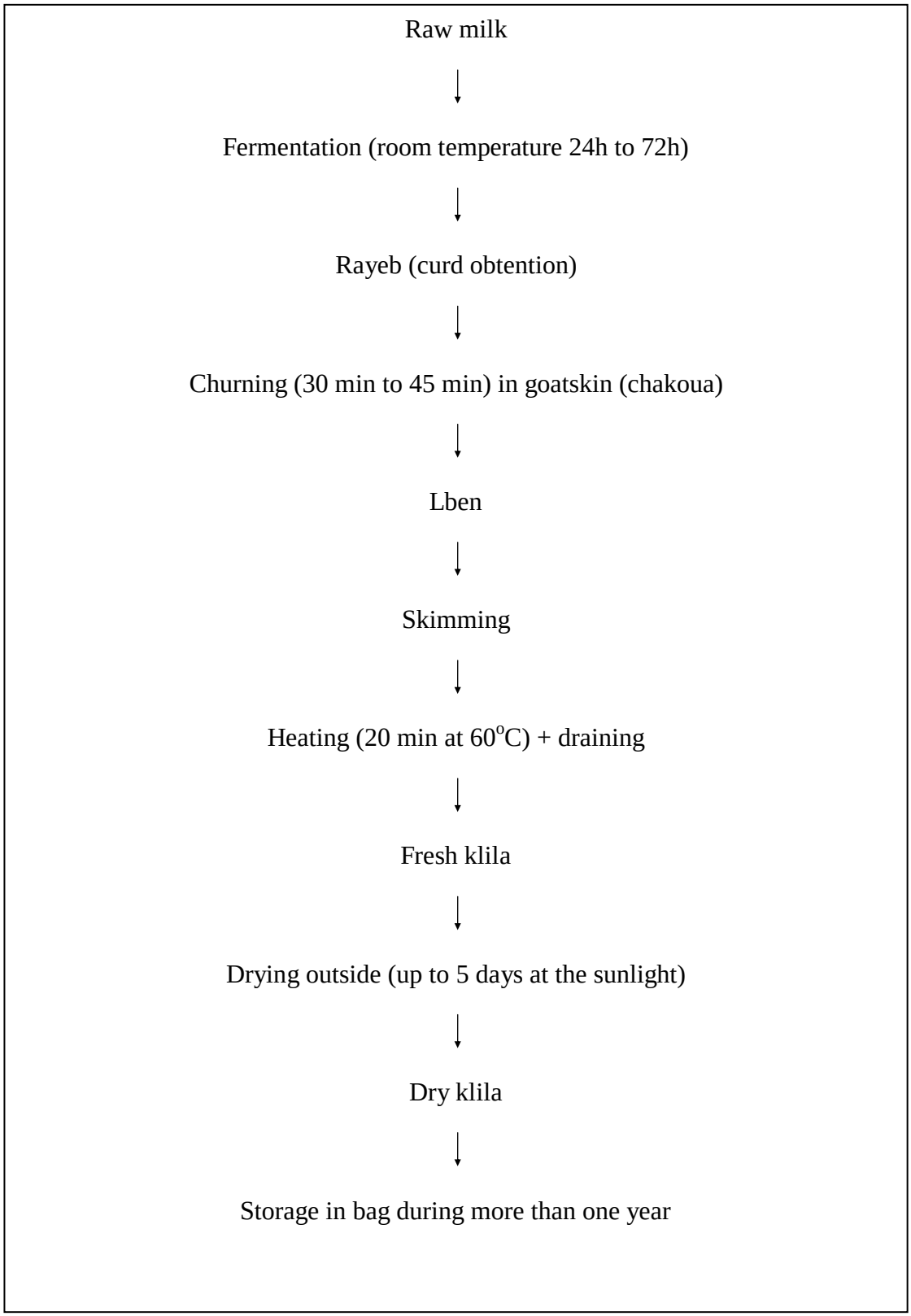


Figure.I.3. Diagram of Klila cheese making **(Benamara *et al.*, 2016)**

I.3. Microencapsulation

I.3.1. Definition

Microencapsulation is defined as a technology of including sensitive ingredients (solid, liquid or gaseous) within several matrices since the ingredients are entrapped or completely surrounded by the protective matrices **(Deprisco and Mauriello, 2016)**. The encapsulated substance is named core material, active agent, filler agent, internal phase, or payload phase. The encapsulating substance is a coating membrane, shell, carrier or wall material, external phase or matrix **(Zuidam and Nedovic, 2010)**.

Hence, microencapsulation of probiotics represents the incorporation of probiotic bacteria into a specific material in order to preserve probiotic viability, reduce cell loss, resist harsh environmental conditions and permit their release under specific conditions **(Deprisco and Mauriello, 2016)**. According to bead size, two types of encapsulation were found: macroencapsulation with beads diameter ranging from millimeters to centimeters and bacterial cells will normally grow on the beads surface due to depletion in nutrient diffusion efficiency in depth of more than 300-500 μm as well as toxic metabolites accumulation in the center of the beads. In case of microencapsulation, beads diameters are between 1 and 1000 μm. Microcapsules are mechanically more robust than macrocapsules **(Park and Chang, 2000)**.

A microcapsule is a sphere with diameter ranging between few microns to 1 mm. Three types of capsules were obtained, reservoir type, matrix and coated matrix type (**figure I.4)**. In the matrix type the active agent dispersed over the encapsulating material. In the reservoir type a layer around the core material was found (also called capsule). The third type is called coated matrix, it is a combination of the previous types, where the active agent is a capsule covered by an additional layer **(Lakkis, 2007)**. The most important properties of microcapsules are the water-insoluble to maintain their integrity in the food matrix and in the upper part of the GI tract and finally, ability of cells release during the intestinal step **(Picot and Lacroix, 2004; Ding and Shah, 2007)**.

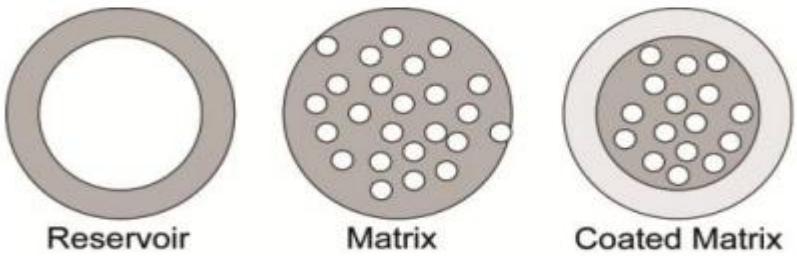


Figure I.4. Types of capsules **(Zuidam and Nedovic, 2010).**

I.3.2. Encapsulating materials

To be used for probiotic encapsulation, the matrix or the biopolymer must have the following criteria: it must be food grade, and it must possess adequate chemical and physical characteristics ensuring protection of bacteria inside. It is important to note that the final morphological and functional characteristics of probiotics are dependent on the type of matrix and on the technique used **(Deprisco and Mauriello, 2016)**.

Wide varieties of polymers were used for encapsulation by researchers, thus, it is important to choose the appropriate polymer according to the objective of encapsulation. The most commonly used food-grade biopolymers include proteins (whey proteins and caseins and gelatin) and carbohydrates (alginate, starch, gums, carrageenan and xanthan) **(Etchepare *et al*., 2015)**.

I.3.2.1. Alginate

It is a natural polymer from algal or bacterial origin, structurayl speaking, it is composed of unbranched (1→4)-linked ß-D-mannuronic acid (M) and α -L-guluronic acid (G) residues **(figure I.5)**, it is widely used for bacterial encapsulation, generally as sodium or calcium alginate in the concentration of 0.5-4 %. It has many advantages, mainly it is safe, gel formation is simple and rapid, do not require hard conditions to occur and furthermore, high release in intestinal conditions, however, porosity of gels is the major inconvenient of this gel. **(Krasaekoopt *et al.*, 2004; Gouin, 2004)**.

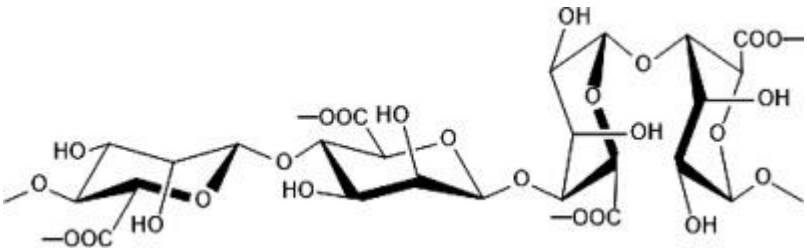


Figure I.5. Chemical structure of alginate **(Lee and Mooney, 2012)**.

I.3.2.2. Gellan gum

A microbial derived polysaccharide (from *Pseudomonas elodea*), it is a result of 4 monomers combination (glucose, glucuronic acid, glucose and rhamnose). Upon cooling, gellan gum produces thermo-reversible gel and gelation temperature depends upon polymer concentration, ionic strength and cation type in the solution **(Vivek, 2013)**.

I.3.2.3. Xanthan gum

Isolated from the bacterium *Xanthomonas campestris*, the basic units of this polysaccharide are glucose, mannose and glucoronic acid. Due to its high gel setting temperature (80-90^{o}C/1h), it is not compatible with viable cells **(Garcia *et al.*, 2000)**.

I.3.2.4. Chitosan

It is a polysaccharide derived from insect cuticles, membranes of fungi and crustacean shells, the basic units are glucosamine, as presented in **figure I.6 (Zargar *et al.*, 2015)**. It is well known for film forming, this is why it is preferred as coating material surrounding a capsule and avoided as encapsulating one since it affected bacterial cells viability negatively with direct contact **(Krasaekoopt *et al.*, 2004)**.

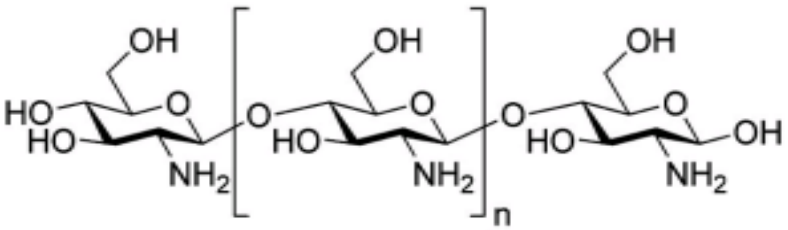


Figure I.6. Chemical structure of chitosan **(Mahapatro and Singh, 2011)**.

I.3.2.5. Cellulose Acetate Phtalate (CAP)

It is a chemically inert polysaccharide, the reason behind its use in bacterial encapsulation, it is also non toxic and highly resistant to acid environment. CAP is widely used as a coating agent. It is used for controlling drug release in the intestine due to its safe nature and because it is physically inert **(mortazavian *et al.*, 2008)**. The encapsulation of probiotic bacteria using CAP provides good protection for microorganisms in simulated GI conditions (**Chopde *et al.*, 2014)**.

I.3.2.6. Starch

Polysaccharide where monomers are glucose units, molecules linked together with α -D-(1-4) and/or α -D-(1-6) linkage. The specific feature of starch (resistant starch) is its ability to be fermented in colon since it resists pancreatic enzymes and as a result released in large intestine. Hence, resistant starch has a double function; it is used as prebiotic by probiotic cells and serves as a carrier for them **(Mortazzavian *et al.*, 2008)**.

I.3.2.7. K-carrageenan

It is a natural polysaccharide associated to sulphate groups as showing **figure I.7 (Kariduraganavar *et al.*, 2014)**. It is used as a thickener and as a stabilizer agent in foods; nevertheless, it is not assimilated by the human body (fiber). It is used for cells encapsulation at temperature of 40-50^{o}C, however, it is not suitable to be used for gastrointestinal stress resistance **(Krasaekoopt *et al.*, 2003; Chen and Chen 2007)**.

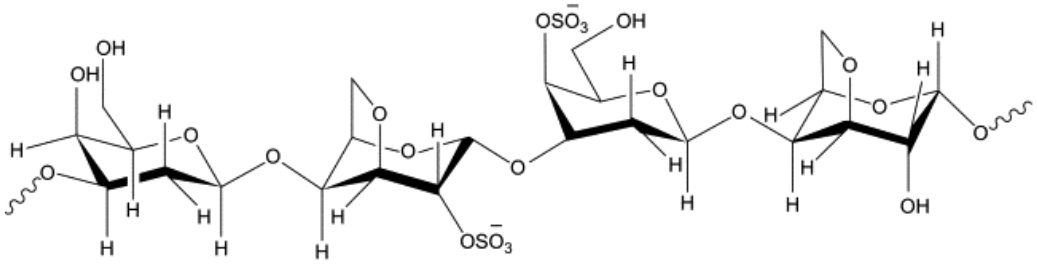


Figure I.7. Structure of k-carrageenan **(Kariduraganavar *et al.*, 2014)**.

I.3.2.8. Gum Arabic (Acacia gum)

It is a natural polysaccharide extracted from acacia trees and it is composed of various monomers (**figure I.8) (Mariod, 2018)** in a complex manner with branched chains, it is known by its low viscosity and high water solubility. Gum Arabic is a complex highly branched polysaccharide, consist of mixed calcium, magnesium and potassium salt of polysaccharides acid. Its main chain is composed of 1,3-linked β-D-galactopyranosyl units and the side chain (2 to 5 1,3- linked β-D-galactopyranosyl) units are joined to the main chain by 1,6- linkages. Both the main and side chains comprise α-Larabinofuranosyl, α-L-rhamnopyronosyl, β-D-glucuronopyranosyl and 4-O-methyl-β-D-glucuronopyranosyl **(Ali *et al.*, 2009)**.

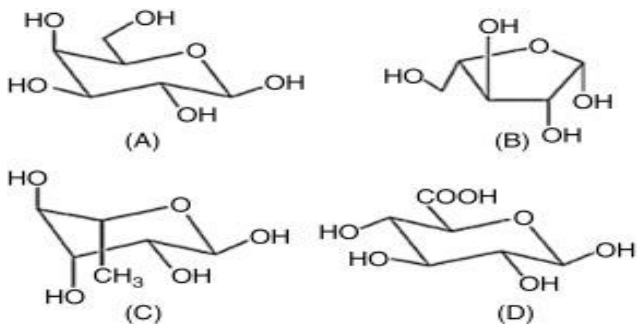


Figure I.8. Structure of gum Arabic **(Mariod, 2018)**.

I.3.2.9. Locust bean gum

The structure of this polysaccharide is presented in **figure I.9,** the principal units are galactomannan while mannose and galactose contents have been reported to be about 73-86% and 27-14%, respectively (mannose:galactose ratio are of approximately 4:1) joined to form a linear chain of (1 → 4)-linked β-D-mannopyranosyl units (mannopyranose) with(1 → 6) linked α-D galactopyranosyl residues (galactopyranose) as side chains **(Menieur *et al.*, 2014)**. It is extracted from the seed of the locust/carob tree (*Ceratonia siliqua* (*L.*) *Taub*) of the family *Leguminosae* **(Menieur *et al.*, 2014)**. Locust bean gum is a cationic, natural polysaccharide and used to improve the stability of alginate beads **(Cheow *et al.*, 2014)**.

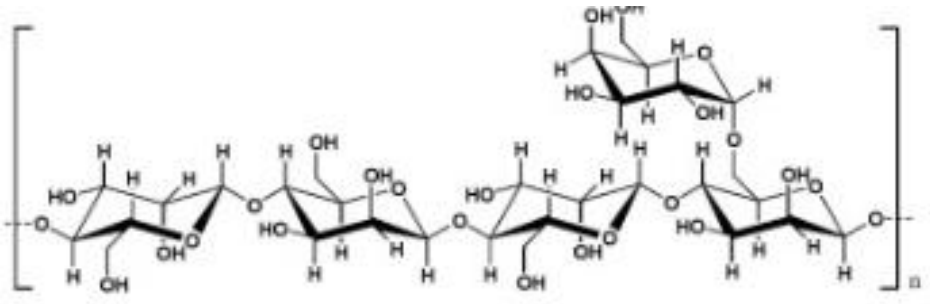


Figure I.9. Chemical structure of locust bean gum, where *n* indicates the number of galactomannan unit repeats **(Menieur *et al.*, 2014)**.

I.3.2.10. Gelatin

Gelatin is another type of polymers used for bacterial cells encapsulation, it is a protein with amphoteric property which has the ability to combine with polysaccharides and form efficient capsules **(Krasaekoopt *et al.*, 2003)**. Gelatin, a biodegradable protein material derived from the partial collagen hydrolysis, was the primary commercial choice as a wall material due to its excellent water solubility, emulsifying and thickening capacity, and high crosslinking activity due to the presence of primary amino groups **(Shu *et al.*, 2006)**.

I.3.2.11. Whey and Milk proteins

Whey proteins are a mixture of globular proteins isolated from whey, the liquid material created as a result of the production of cheese. All of them are characterized by their high ability to form gels and to probiotic encapsulation and delivery due to their physicochemical properties mostly biocompatibility **(Liveney, 2010)**.

I.3.2.12. Pectin

Pectin is a heteroploysacchride mainly extracted from fruits. It is used as gelling agent in food, in medicines and as a source of dietary fibers. It remains intact in the stomach and in the small intestine; hence, it is used solely or in combination with other matrices to encapsulate probiotic bacteria **(Gebara *et al.*, 2013)**.

I.3.2.13. Chickpea protein

It is a vegetable encapsulating material with excellent functional and nutritional properties with low allergic reactions. This protein is constituted of types of salt-soluble globulin-legumin and vicilinttributes. It offered good protection for encapsulated cells against gastric conditions and it is also found that it is a good carrier for applications in food **(Klemmer *et al.*, 2011; Wang *et al.*, 2014)**.

I.3.3. Encapsulating techniques

A wide range of encapsulation techniques were described, discussed in different reviews, as well as encapsulating materials, however, each technique has advantages and disadvantages and by consequence, its effectiveness is highly related to the availability, to cost, and to biocompatibility. Among the technologies applied: emulsion, spray drying and extrusion are the most studied and applied on both laboratory and industrial scale. New technologies as complex coacervation and vibrating technology seem to be efficient **(Bosnea *et al.*, 2014; De Prisco *et al.*, 2015)**.

I.3.3.1. Extrusion

Extrusion is a simple and low cost procedure that makes minimal injuries where viability of probiotics is efficiently maintained **(Mortazavian *et al.*, 2007)**. It is an easy technique where a mixture of probiotic cells and encapsulating matrix was injected into a hardening solution through an extruder (syringe) as shown in **figure I.10**. The crosslinking occurred with calcium ions **(Krasaekoopt *et al.*, 2004)**. Mostly, alginate and calcium chloride concentration ranges from 0.5% to 4% and from 0.05 to 1.5 M, respectively, and the beads size ranges from 2 to 3 mm in diameter which mostly depends upon the distance between syringe and hardening solution, polymer type, viscosity, concentration and mainly diameter of the extruder orifice **(Solanki *et al.*, 2013)**.

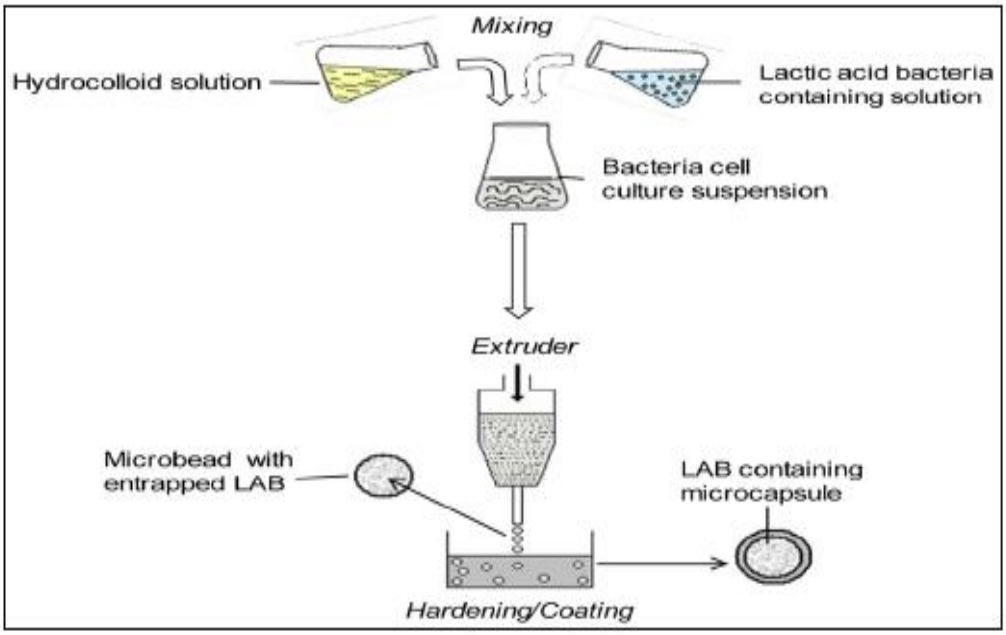


Figure 1.10. The encapsulation process of probiotics by extrusion technique **(Feucht and Kwak, 2013)**.

I.3.3.2. Emulsion

It is a chemical interaction between two phases, continuous (soybean, sunflower, canola, olive oil..) and discontinuous (cell polymer), calcium chloride is also needed as a hardening agent, beads sizes varied between small and large, they are encapsulating material, concentration and viscosity and agitation rate dependent (**figure I.11) (Chávarri *et al.*, 2012)**.

While emulsion technique is characterized with many advantages: high viability of cells and the ease of the scale-up; it has also some disadvantages: the large size range and shape in addition to the cost (expensive method). Alginate, carrageenan, and pectin are widely used in emulsion **(Burgain *et al.*, 2011)**.

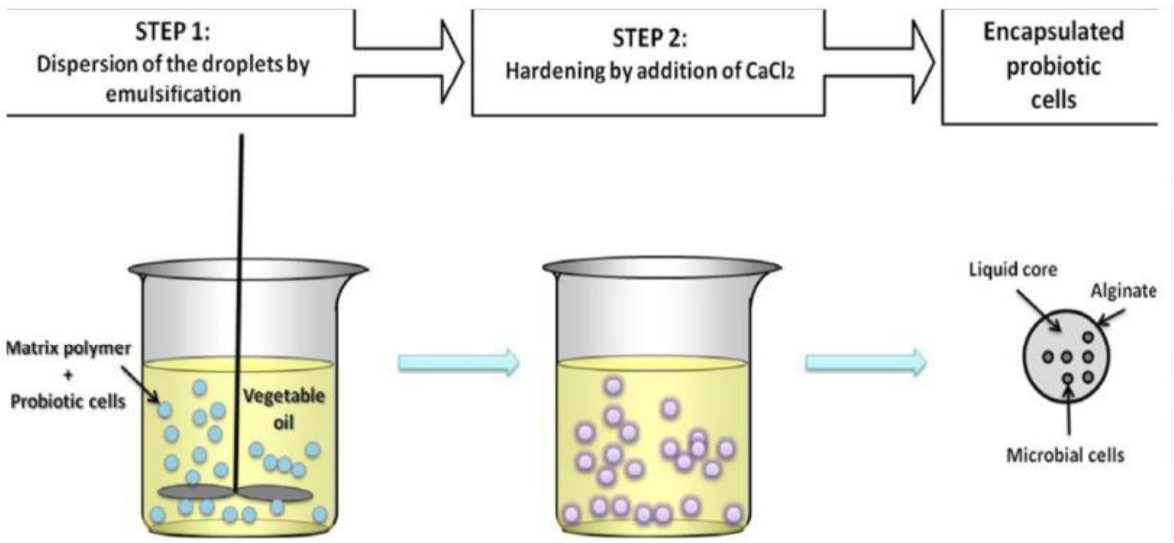


Figure I.11. The encapsulation process of probiotics by emulsion technique **(Burgain *et al.*, 2011)**.

I.3.3.3. Spray drying

It is so called because the final obtained capsules appeared as a dry powder, the principle consists of the addition of a mixture of bacterial cells and encapsulating material to a drying gas where atomization occurred (**figure I.12) (Chávarri *et al.*, 2012)**.

Comparing to the other techniques, small capsules are obtained, it is considerably cheap, rapid, widely scale-up and so adequate for industrial use, however, cell viability is reduced as consequence of the use of high temperature which is the limiting factor for this technique. The common matrices used are: polysaccharides, proteins, skim milk, gelatin, starch, gum Arabic **(Burgain *et al.*, 2011)**.

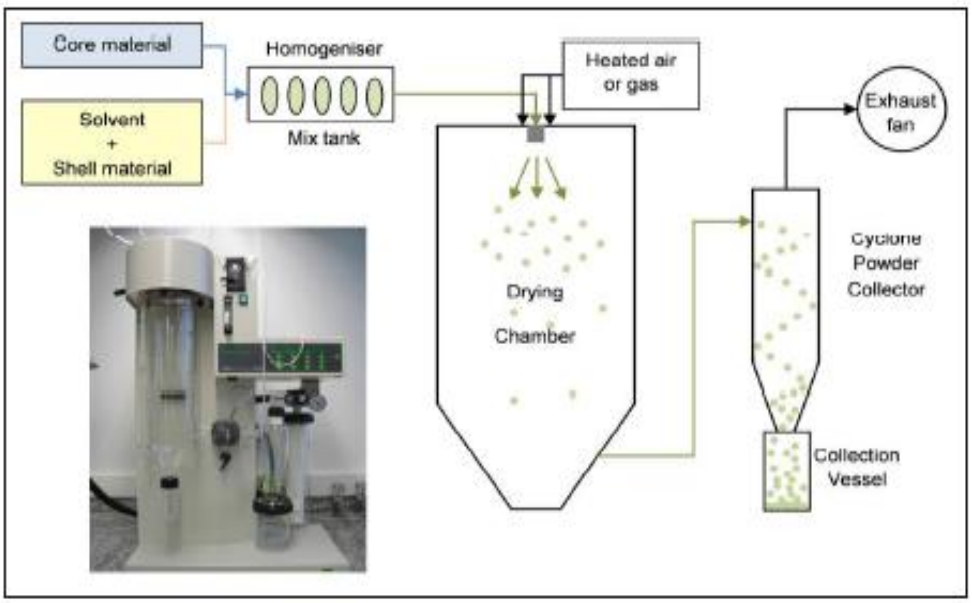


Figure I.12. Encapsulation process of probiotics by spray drying **(Chávarri *et al.*, 2012)**.

I.3.3.4. Coacervation

This microencapsulation technique utilizes phase separation of one or more incompatible polymers from the initial coating polymer solution under specific temperature, pH or composition of the solution. The incompatible polymer is added to the coating polymer solution and the dispersion is stirred. Changes in the physical parameters lead to the separation of incompatible polymer and deposition of dense coacervate phase surrounding the core material resulting in the production of microspheres **(John *et al.*, 2011)**. It is a promising technique since it has a good encapsulation capacity and controlled liberation of the core material from the microspheres, conferring with a remarked resistance against harsh conditions **(Oliveira *et al.*, 2007)**.

I.3.3.5. Spray chilling/cooling/congealing

It is a technique with the same principle as spray drying, instead of injection of hot air, the cold air is injected, and the beads formed **(Champagne and Fustier, 2007)**.

I.3.3.6. Freeze-drying

Another encapsulating technique where cells are first frozen and then dried by sublimation of the solvent, normally water under high vacuum. The disadvantage of this method is the formation of crystals which causes the cell destruction and the stress condition of high osmolarity. To solve the problem of osmolarity, several cryoprotectants have been used in order to keep viability of microbial cells during dehydration, such as glucose, trehalose, maltodextrine, skim milk powder and whey protein. They permit also the adaptation of microbial cells to the environment **(Mokarram *et al.*, 2009; Basholli-Salihu *et al.*, 2014)**. These cryoprotectants accumulate within cells, decreasing the osmotic difference between the internal and external cell environments **(Lopez-Rubio *et al.*, 2009; Martın *et al.*, 2015)**.

I.3.4. Factors affecting microencapsulation effectiveness

Sornplang and Piyadeatsoo (2016) summarized in their review the factors to which the microencapsulation is dependent: conditions of processing factors, bacterial cell suspension concentration, type of encapsulating material, interactions between bacterial cell and capsule, surrounding factors, capsule material coating and processes, encapsulating material concentration and diameter of the obtained capsules.

Many authors underline the industrial feasibility of probiotic microencapsulation **(Burgain *et al.*, 2011; Chavari et al., 2012; Feucht and Kwak, 2013)**. Among different techniques,

spray drying and coacervation are considered by **Chavarri *et al.* (2012)** as the cheapest technique, even though the former is rarely applied because of thermal cell inactivation and the scale up of the latter is quite arduous. Instead, the same authors report that the scale up of vibrational extrusion is relatively simple. Similar opinion was expressed by **Burgain *et al,* (2011)**, who referred to extrusion as a simple and low cost technique. On the other hand, microencapsulation can theoretically reduce the cost linked to the production of biomass for probiotication of food. In fact, the main goal in applying microencapsulation is the higher cell recovery at the end of food process and consumption leading to use lower amount of encapsulated cells to achieve the same probiotic effect of free cells **(Deprisco and Mauriello, 2016)**.

I.3.5. Applications of microencapsulation

The encapsulation technique has a large spectrum of applications, and this is related to their advantages: production of quality food products, achieving new methods in food manufacture, increasing probiotics viability against harsh conditions, maintain food safety during fermentation, and improving functional properties of product **(Sornplang and Piyadeatsoo, 2016)**.

I.3.6. Probiotics in food products

The concept of food having medicinal value is called functional foods. Designer foods, medicinal foods, nutraceuticals, therapeutic foods, superfoods, foodiceuticals, medifoods are different names of functional foods, foods that have been modified in some ways to become functional **(Shah, 2007)**. Selection of food systems to deliver probiotics and microencapsulation use are important in developing functional probiotic foods since food matrices are vehicles of transport to gastrointestinal tract and since microencapsulation can also improve the viability of probiotic in some food matrices **(Ranadheera *et al.*, 2010; Ruiz and Segura-Campos, 2017)**. This is convinced by the study of **Godward and Kailasapathy (2003)** which tested the impact of addition of encapsulated cells to defined food matrices and concluded that the addition enhanced some foods whereas it is not necessary for others proving the importance of selection of food as matrix carrier.

I.3.6.1. Dairy products

Literature highlighted that dairy products are the preferred vehicles for delivering probiotic bacteria to the human gastrointestinal tract. The most frequently used matrices are cheese, yoghurt, ice cream and other dairy products **(Kent and Doherty, 2014)**.

I.3.6.1.1. Yoghurts

In spite the presence of considerable amount of organic acids and the low pH, yoghurt and other fermented milk beverages are the principal food carrier of probiotics, this is due to their nutritional value and their compliance with worldwide dietary habits acceptance by consumers **(Sanders, 2008b)**. The incorporation of probiotic living cells in yoghurt enhances its therapeutic value **(Chen and Chen, 2007)**. However, there is poor level of probiotic viability in yoghurt because of the low pH (from 4.2 to 4.6). Studies showed that the use of encapsulated probiotic bacteria was better for their survival. Furthermore, the incorporation of probiotic cells into yoghurts could be carried out without making many modifications from the traditional process **(Kailasapathy, 2006)**.

I.3.6.1.2. Cheese

Because having specific criteria; low moisture content, presence of salt, starter cultures competing for nutrients and developing acid and flavor during the maturation, cheese is used as a vehicle for probiotic delivery. For example, Cheddar cheese presents the advantage of being a good carrier of probiotic microorganisms. In addition, its good buffering capacity and its relatively high fat content may offer a protection to probiotic bacteria against enzymatic degradation and acidic environment of the GI tract **(Dinakar and Mistry, 1994)**.

I.3.6.1.3. Ice cream and frozen dairy desserts

These products have the advantage to be stored at low temperatures, which makes them less exposed to abusive temperatures and so they have higher viability at the time of consumption. It is not easy to incorporate probiotic microorganisms into frozen desserts because of high acidity in the product, high osmotic pressure, freeze injury and exposure to the incorporated air during freezing **(Chen and Chen, 2007)**. The introduction of probiotic bacteria in an encapsulated form into frozen desserts may overcome these difficulties and could produce useful markets and health benefits **(Chen and Chen, 2007)**.

I.3.6.2. Non-dairy products

Fruits, vegetables, legumes and cereals are examples of non dairy products. Because they are rich on minerals, vitamins, dietary fibers, and antioxidants, fruits and vegetables are considered as good matrices **(Yoon *et al.*, 2006; Antunes *et al.*, 2013**). Moreover, the increasing demand on products with low cholesterol content and free from animal derivatives and milk allergens makes non dairy products an interesting alternative food carriers **(Espedes *et al.*, 2013)**. Fruit juices are considered acidic media for probiotics, however, for their pleasant taste and acceptability by consumers, fruit juices could serve as a probiotic food

category with easy and steady assumption, in addition, they would be the next food category where the healthy probiotic bacteria will make their mark **(Prado *et al.*, 2008)**.

I.3.6.3. Other food carriers

Other foods can serve as probiotic carriers; it is the case of mayonnaise and vinegar, where, encapsulated bifidobacteria were incorporated and exhibited good viability. The potential use of microencapsulation to protect the cells in meat products were also investigated **(Muthukumarasamy and Holley, 2007)**. Moreover, dry fermented sausages enriched with encapsulated probiotics did not affect sensory properties of the product. The enrichment of meat products with probiotic bacteria concerns also their use as protective culture in order to achieve an antimicrobial effect against spoilage and pathogenic population **(Gao *et al.*, 2014; Sidira *et al.*, 2014)**. The addition of probiotic cultures in bakery goods (bread, biscuits, cakes and pastries) is still little investigated **(Malmo *et al.*, 2013; Deprisco and Mauriello, 2016)**.

Materials and methods

The work took place in the Laboratory of Molecular Toxicology, Department of Applied Microbiology and Food Sciences at the University Mohamed Seddik Benyahia of Jijel (Algeria) between the years 2013 and 2018. A part of this work was conducted at the Department of Agriculture, University of Naples Federico II, Portici, Napoli, Italy. All experiments were conducted in duplicate.

II.1. Bacterial isolates

Lactic acid bacteria (LAB) used in this study were isolated from the Algerian traditional fermented cheese 'Klila' from Ouergla City. Samples (dried Klila) were transported to the laboratory for bacterial isolation.

Pathogenic bacteria used for antagonism activity were *Escherichia coli* ATCC 25922 (UHC, Constantine, Algeria), *Bacillus subtilis*, *Salmonella* sp., *Staphylococcus aureus* and *Listeria monocytogenes* (Stock cultures: Laboratory of Biotechnology, Environment and Health, University of Jijel, Algeria).

II.2. Media and chemicals

- MRS broth and agar (CONDA, pronadisa, Madrid, Spain).
- Gibson & Abd-El-Malek medium at pH 6.5 (2.5 g yeast extract, 50g glucose, 100 ml tomato juice, 50 ml skimmed milk, 200 ml nutrient agar). Sterilization was achieved by tyndalisation 3 times 20 mn at 100^{o}C.
- Phosphate Buffer Saline (PBS) at pH 7.4: two solutions were prepared, A and B, then they were mixed together (Solution A: 13.8g of NaH_2PO4 in 200ml of distilled water. Solution B : 63.6g of $Na_2\ HPO_4$ in 800ml of distilled water).
- Mueller Hinton agar (Pasteur Institute, Algeria).
- Commercial pineapple beverage (N'Gaous, Algeria). The composition is (water, sugar, concentrate of pineapple, concentrate of apple, stabilizers "pectin, carob gum" (1.14g/l), colorant, aroma, carotene, acidity regulator, citric acid, antioxidant, ascorbic acid "vitamin C").
- Commercial strawberry beverage (TOUDJA, Bejaia, Algeria). The composition is (water, sugar, concentrate of fruit minimum 20 "juice and pulp of strawberry, carrot concentrate", natural aroma, food additives: citric acid, ascorbic acid, carboxymethyl cellulose, glycosides).

II.3. Isolation and purification of lactic acid bacteria

One g of Klila was weighed and added to 9 ml of normal saline solution (0.85% NaCl w/v), then serial dilutions were made eight (08) folds. A volume of 0.1 ml of the appropriate dilutions was homogenously distributed on MRS agar surface and the plates were incubated at 37°C for 48h under anaerobic conditions. To prevent yeasts growth, MRS agar was supplemented with 0.14% sorbic acid. Well separated colonies were chosen randomly and carefully and inoculated in MRS broth and incubated for 24h, again these selected colonies were streaked on MRS agar and incubated for 24h at 37°C. This step was repeated till obtained colonies appeared with the same color, the same shape and the same size. These colonies were subjected to microscopic examination to confirm their purity.

II.4. Phenotypic characteristics

II.4.1. Gram stain and catalase test

The purified isolates were subjected to the Gram stain where only Gram positive bacilli were chosen, subsequently, these bacteria were subjected to catalase test by the addition of H_2O_2 (10%) to the selected colonies, and only catalase negative (29 isolates) were used for further tests of identification **(Xanthopoulos *et al.*, 2000)**.

II.4.2. Production of gas (CO_2)

To test the ability to produce gas from glucose and hence to determine the profile of fermentation homo or heterofermentavie, the method described by **Guiraud (1998**) was followed, the medium Gibson & Abd-El-Malek was inoculated with the isolates and the top of the medium was covered with paraffin than tubes were incubated at 37°C for 7days, any movement of this layer was due to gas production and hence bacteria are heterofermentative.

II.4.3. Fermentation of carbohydrates

10 µl of each isolate was inoculated in 1 ml of the bromocresol purple medium to which was added the selected sterile sugar (carbohydrate) then, some drops of paraffin were added to the top of each medium to form a protecting upper layer and thus anaerobic conditions were created, incubation was carried out for 24h at 37°C. The following sugars were used: glucose, D-xylose, cellobiose, levulose, sorbose, trehalose, mannose, inositol, galactose, sucrose and raffinose. Results were positive if yellow color appeared **(Giuraud, 1998)**.

II.4.4. ADH test

Following the method of **Hariri *et al.* (2009)**, bacterial isolates were inoculated in arginine Moeller medium then incubated for 48h at 37°C. Apparition of purple color means positive results and so the isolate used arginine while the yellow color means negative results and the isolate was not able to use arginine.

II.4.5. Growth at different temperatures

Growth of the isolated bacteria at different temperature was also carried out. Two temperature values were used; 15°C and 45°C. Inoculated bacteria in MRS broth were incubated for 5 days. Any turbidity of the medium means the presence of the growth **(Leveau *et al.*, 1991)**.

II.4.6. Growth in presence of NaCl

MRS broth was supplemented with NaCl at concentrations of 4% and 6.5% then inoculated with two successive cultures of bacterial isolates. Tubes were incubated at 37^{o}C for 48h. Any turbidity in the medium was considered as growth **(Guessas *et al.*, 2012)**.

II.5. Molecular characterization

II.5.1. DNA extraction

Genomic DNA was extracted using the Insta-Gene matrix (Bio- Rad, Milan, Italy) according to the manufacturer's protocol. Briefly, colonies of each microorganism were suspended in 0.05 M phosphate buffered saline (PBS) pH 7.0 and centrifuged for 1 min at 14,000 rpm. Pellet was washed with PBS then centrifuged again. Supernatant was removed while pellet was resuspended in 200 µl of Insta-Gene matrix and incubated for 30 min at 56°C. The mixture was vortexed for 10s before its transfer to a water bath for 10 min at 100°C. Then, it was centrifuged at 14,000 rpm for 3 min and the resulting supernatant, containing the bacterial DNA, was used for PCR.

II.5.2. Sequencing of 16S rDNA of bacteria

Amplification of the 16S rRNA gene of the isolates was conducted with the following primers: Universal primers (Invitrogen) fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3'). PCR reaction mixture (final volume 50 µl) contained 50 ng of DNA template, 5 µl of 10×buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 25 mM $MgCl_2$, 10 mM dNTPs mix, 50 pM primers and 5 U of Taq Polymerase.

For PCR amplification, the denaturation step was conducted at 95°C for 3 min, followed by 30 cycles of 45 s at 94°C, 45 s at 55°C and 1 min at 72°C each, and by a final extension of 5 min at 72°C. The sequencing was done at Primmsrl (Milan, Italy). BLAST program was used to find DNA similarity. The 16S rRNA partial gene sequences of the studied bacteria have been deposited in the GenBank database under accession numbers. The phylogenetic tree was generated by the MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms **(Kumar *et al.*, 2018).**

II.6. Storage of isolates

The purified bacterial isolates were maintained as frozen stocks in MRS broth supplemented with 30% (v/v) glycerol at -20°C until use when revivification was done both on MRS broth and agar, a check of purity using Gram stain and catalase test was also necessary for each isolate.

II.7. Characterization of some probiotic and technological traits

II.7.1. Technological traits

II.7.1.1. Acidifying ability

Acidifying ability was determined as follow: two successive overnight culture of each isolate (1%) was inoculated into 10 ml of sterile skimmed milk (12%) and then acidity was determined by measuring pH and Dornic acidity at 0h, h3, 6h and 24h of incubation. To measure Dornic acidity, the inoculated skimmed milk was titrated with sodium hydroxide (NaOH) 9N, phenolphthalein was used as indicator until the sample changed color from white to light pink. The needed volume of NaOH for the appearance of the color was registered. The acidity was expressed in **Dornic degree (°D) (1°D = 0.1 g lactic acid/liter and acidity = volume of NaOH x10) (Va´zquez *et al.*, 2013)**. Titratable acidity of lactic acid was calculated according to **FAO (1986)**.

II.7.1.2. Detection of proteolytic activity

To achieve this purpose, MRS agar supplemented with (10%) skimmed milk was used following the method of **De Roissart (1986)** and **Lasagno *et al.* (2002)** with some modifications. After pour plating, sterile WHATMAN discs were placed on the surface, then 100 µl of an overnight culture of each bacterial isolate were deposited on these discs. Plates were incubated at 37°C for 24h. The presence of clear zones around the colonies was recorded as positive activity

II.7.1.3. Detection of lipolytic activity

An overnight culture of the tested strains grown at 37°C in MRS broth was used. Bacterial isolates were streaked on MRS agar supplemented with (1%) olive oil or 1% (v/v) of tributyrin as substrate **(Jini *et al.*, 2011)**. Activity was observed as a detectable zone of hydrolysis after 48 h of incubation at 37°C.

II.7.1.4. Texturing activity

This property was determined on MRS agar supplemented with high sucrose concentration (up to 20 g/l), a fresh culture (18h) of each isolates was streaked on the surface of medium and incubated for 24h at 37°C. Positive test was determined by the appearance of big glamour colonies **(Leveau *et al.*, 1991)**.

II.7.2. Probiotic traits

II.7.2.1. Antibacterial activity

The agar spot method described by **Benerji *et al.* (2013)** was used to realize this test with the following indicator strains, *Escherichia coli* ATCC 25922, *Staphylococcus aureus*, *Salmonella* sp., *Bacillus subtilis*, and *Listeria monocytogenes*. Ten (10) µl of two successive overnight cultures of each isolate were spotted on the surface of MRS agar and incubated for 24h at 37°C, then 10 ml of Muller Hinton supplemented with 1 ml of standardized suspension of each pathogen (10^6 CFU/m1) was pour plated on the previous prespotted bacteria. The inhibition diameters were measured after 24h incubation at 37°C.

II.7.2.2. Resistance to antibiotics

The disk diffusion method was adopted following the procedure of **Liasi *et al.* (2009)**. Fresh cultures of the isolates were standardized and swabbed on the surface of the MRS agar. The five tested antibiotics: Penicillin G (10 µg), Amoxicillin (25 µg), Colistin sulfate (50 µg), Gentamycin (10 µg) and Streptomycin (30 µg) were placed on the surface of the inoculated plate. After incubation for 48 h at 37ºC, the diameter of the inhibition zones was measured. Bacteria were classified as sensitive (diameter of inhibition zone is ≥ 21 mm), intermediates (diameter of inhibition zone is 16-20 mm) and resistant (diameter of inhibition zone is ≤ 15 mm).

II.7.2.3. Acid tolerance

This test was performed as described by **Pieniz *et al.* (2014)**. Two successive overnight cultures of the twenty nine (29) isolates were centrifuged at 6000 rpm for 15 min; the pellet was washed twice with sterile normal saline and resuspended in PBS buffer (pH 7.5). An

acidified PBS at pH 03 (adjusted with HCl) was inoculated with the obtained bacterial suspensions then incubated at 37°C. Viable count was performed on MRS agar both at 0 time (t0) and after 2h of incubation. The ratio between the two counts was considered as viability (%).

II.7.2.4. Resistance to simulated gastrointestinal conditions

In the purpose of testing the viability of LAB in simulating gastric and intestinal (SGI) conditions, two successive cultures of fourteen (14) isolates were centrifuged at 6000 rpm for 15 min, the pellet was washed twice with sterile normal saline and then resuspended in PBS buffer. One (1) ml of each bacterial suspension was transferred to 9 ml of gastric simulated juice and 9 ml of simulated intestinal juice and then incubated for 2h at 37°C. Serial dilutions were performed for both inoculated simulated juices and viable counts were determined. The percentage of viability between the end and the beginning of incubation was determined (The simulated gastric juice (SGJ) composition was: 0.3% (w/v) pepsin, 0.5% (w/v) NaCl, 0.22 % (w/v) KCl, 0.12% (w/v) $NaHCO_3$ and 0.022% (w/v) $CaCl_2$; (pH 2.5). Simulated intestinal juice (SIJ) composition was: 0.1% (w/v) pancreatin and 0.128% (w/v) NaCl, 0.023% (w/v) KCl, 0.64% (w/v) $NaHCO_3$ and 0.5% (w/v) bile salts, pH was adjusted to 7.5).

The two strains *Lb. casei* B1 and *Lb. plantarum* Q18 were selected for further work.

II.7.2.5. Bile salts tolerance

Overnight cultures of *Lb. casei* B1 and *Lb. plantarum* Q18 were centrifuged at 6000 rpm for 15 min, the pellet was washed twice with sterile normal saline and then resuspended in PBS buffer, and the initial count was determined to give about 10^{10}CFU/ml. These cultures were inoculated in bile salts solution at 0.3% for 8 h then viable count was carried out. The percentage of viability between the end and the beginning of incubation was determined **(Kaewnopparat *et al.*, 2013)**.

II. 7.2.6. Hemolysis test

The production of hemolysin was determined according to the method described by **Li *et al.* (2014)**. The selected strains were streaked on blood agar (5% horse blood), then incubated for 48h at 37°C. The presence or the absence of clear zones around the colonies was interpreted as beta-hemolysis (positive) or gamma-hemolysis (negative) activity, respectively.

II.7.2.7. Cell surface hydrophobicity

Hydrophobicity test was conducted following the method described by **Iyer (2010b)**. After centrifugation of the fresh cultures, at 6000 rpm for 15min, the obtained pellet was washed twice with sterile urea-magnesium sulfate buffer, and resuspended within the same buffer to obtain the initial optical density approximately 1 at 450 nm. Three ml of each suspension was added to 0.6 ml of xylene and then incubated for 10 min at 37°C. The mixture was then vortexed and incubated again for 20 min. OD of the aqueous phase was measured at 450 nm, and hydrophobicity was calculated using the following formula:

$$\% \text{ Hydrophobicity} = [OD_i - OD_f / OD_i] \times 100$$

OD_i: initial optical density at t=0h,

OD_f: final optical density

II.7.2.8. Adhesion to intestinal epithelial cells

Adhesion to epithelial cells was determined using the method described by **Kos *et al.* (2003)**. Intestinal cells used were obtained from poultry ileum. This intestinal poultry ileum was washed three times with PBS and a final intestinal cells concentration of 10^4 CFU/ml was used. Bacterial cells suspensions were used at concentration of 10^8 CFU/ml approximately; they were first centrifuged at 6000 rpm for 15min, washed with sterile normal saline and resuspended in PBS. A mixture of equal volumes (1 ml/1 ml) (intestinal cells/bacterial cells) was incubated for 30 min at 37°C. Result was positive when attached cells were more than 10 bacterial cells/ epithelial cell and thus using microscopic observation (x100) after their staining with crystal violet.

II.7.2.9. Autoaggregation

Two successive overnight cultures of *Lb. casei* B1 and *Lb. plantarum* Q18 were centrifuged at 6000 rpm for 15min separately, washed and resuspended in PBS, optical densities at 600 nm were taken and considered as initials. Optical densities of the undisrupted upper layer were measured again after 3h and 5 h following the procedure of **Balakrishna (2013)**.

$$\text{Autoaggregation (\%)} = 1 - (OD_t / OD_i) \times 100$$

OD_t: optical densities after 3h and 5h.

OD_i: initial optical density at t0

II.8. Encapsulation of *Lb. casei* B1 and *Lb. plantarum* Q18

II.8.1. Encapsulation with sodium alginate

The method described by **Sheu *et al.* (1993)** with some modifications was used. An overnight culture of bacteria grown on MRS broth was centrifuged for 10 min at 6000 rpm. The obtained cell pellets were then washed with 10 ml of normal saline (0.9%) and were resuspended in the same volume of PBS. Cell concentration was adjusted to approximately ~10^{10}- 10^{11} CFU/ml. This cell suspension was mixed with an autoclaved solution of 2% (w/v) sodium alginate, and the number of beads corresponding to 1 ml was counted. This procedure was repeated 5 times.

II.8.1.1. Tolerance to acid pH

One ml of the bacterial suspension mentioned above (free cells) and 50 beads of encapsulated bacteria (immobilized cells) were added to 9 ml of normal saline (0.9% NaCl) separately at three pH values, 2, 4 and 7 according to the method described by **Bosnea *et al.* (2014)**. The incubation was carried out at 4 °C for 3 h, 7 and 14 days for evaluation of the applied stress and 1 ml of the previous solutions was transferred to 9 ml of PBS and after serial dilutions viable cell count was determined on MRS agar.

II.8.1.2. Tolerance to NaCl

Three saline solutions with different salt concentrations were used (3%, 6% and 9%), and the same procedure used for the pH stress was followed. The mixture was incubated at 4°C for 3 h, 7, 14, 21 and 28 days **(Bosnea *et al.*, 2014)**. Cell count was determined as previously described.

II.8.1.3. Storage in strawberry beverage

To evaluate the effect of strawberry beverage composition on the viability of both strains, one ml of free and encapsulated cells was introduced separately in tubes containing 9 ml of a commercial strawberry beverage (TOUDJA, Bejaia, Algeria). The tubes were stored at 4^{o}C, and viable count on MRS agar was evaluated at 0, 3 h, 7 and 14 days. The initial cell number in the beverage was approximately 2×10^{10} CFU/ml for both strains **(Nualkaekul *et al.*, 2013)**.

II.8.2. Encapsulation of *Lb. plantarum* Q18 with sodium alginate using vibrating technology and coating with chitosan

Lb. plantarum Q18 was also encapsulated with the vibrating technology using the encapsulator B-395 Pro equipped with an 80 mm nozzle and a syringe pump (BÜCHI Labortechnik, Flawil, Switzerland). Sodium alginate 2% was the only polymer used; the same

procedure described above was followed; only the syringe is replaced with the encapsulator (**Deprisco *et al.* (2015)**.

The chitosan solution was prepared based on the method described by **Chavari *et al.* (2012)**. Chitosan solution (0.8%) was prepared by dissolving 8 g of chitosan in 1000 ml of distilled water. The pH was adjusted to 3.2-3.4 with citric acid. The solution was autoclaved at 120°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 beads. Such microcapsules were then separated by filtration, and then washed with distilled water.

II.8.2.1. Viability under simulated gastrointestinal conditions

The assay was performed according to **Vizoso *et al.* (2006)**. Viable cell counts were monitored during exposure to SGI conditions, after 2 h to assess the gastric transit tolerance and then after 4 h to evaluate the intestinal transit tolerance. The simulated gastric juice (SGJ) was prepared using 0.3% (w/v) pepsin (SIGMA, Milan, Italy), 0.5% (w/v) NaCl, 0.22% (w/v) KCl, 0.12% (w/v) $NaHCO_3$ and 0.022% (w/v) $CaCl_2$; and was acidified with HCl (0.1M) to pH 2.5. Simulated intestinal juice (SIJ) was prepared with 0.1% (w/v) pancreatin and 0.128% (w/v) NaCl, 0.023% (w/v) KCl, 0.64% (w/v) $NaHCO_3$ and 0.5% (w/v) bile salts (SIGMA, Mian, Italy), pH was adjusted to 7.5. Both solutions were filtered using 0.22 µm membranes filters. Free and encapsulated cells were exposed to simulated GI conditions and their viability was calculated for different times (0, 2 and 4 h).

II.8.2.2. Capsules morphology

The LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes, Eugene, Oregon, USA) was used to prepare the obtained *Lb. plantarum* Q18 capsules by vibrating technology for visualization with fluorescent microscopy, images were taken with photographic camera (Nikon D7000) (**Malmo *et al.*, 2013)**.

II.8.3. Encapsulation of *Lb. casei* B1 and *Lb. plantarum* Q18 using different polymers

An overnight culture of bacteria grown on MRS broth was centrifuged for 10 min at 6000 rpm. The obtained cell pellets were then washed with 10 ml of normal saline (0.9%) and were resuspended in the same volume of PBS. Cell concentration was adjusted to approximately ~10^{10}-10^{11} CFU/ml.

Seven types of polymers (matrices) were used. They were prepared in various combinations in the ratio of (1:1) sodium alginate/chitosan, sodium alginate/k-carrageenan, sodium alginate/glycogen, sodium alginate/gum Arabic, sodium alginate/locust bean gum, sodium alginate /starch and finally sodium alginate alone. The procedure of mixtures preparation was:

- Alginate (2%): 2g of sodium alginate in 100 ml of distilled water.
- Alginate (1%) -k- carrageenan (1%) polymer: 1g of sodium alginate and 1g of k-carrageenan in 100 ml of distilled water.
- Alginate (2%) – glycogen (1%) – glycerol (2.5%) polymer: 2g of sodium alginate, 1g of glycogen and 2.5 ml of glycerol in 100 ml of distilled water.
- Alginate (1%) -locust bean gum (1%): 1g of sodium alginate and 1g of locust bean gum in 100 ml distilled water.
- Alginate (1%)-gum Arabic (1%): 1g of sodium alginate and 1g gum Arabic in 100 ml distilled water
- Alginate (1%) - Starch (1%): 1g of sodium alginate and 1g starch in 100ml distilled water.

The polymeric mixture was mixed with the obtained bacterial suspension then passed through a needle fitted-syringe (2.5 ml) and dropped into 0.5 M calcium chloride solution under slight agitation for 45 min.

II.8.3.1. Storage for different periods

Viability of microencapsulated bacteria during different periods of storage was also studied. The procedure was performed using the method described by **Nualkaekul *et al.* (2013)** and **Deprisco *et al.* (2015)**. Bacterial cells were stored in normal saline and in pineapple beverage for 28 days at 4^{o}C and viable count was performed on MRS agar at 0, 7, 14, 21 and 28 days. Cell load was determined after disruption of microcapsules. Free cells were managed in the same way and used as control sample.

$$\text{Viability (survival)} = \text{Log } CFU_t/\text{Log } CFU_i \times 100$$

CFU_t: Colony Forming Unit after the given period of storage

CFU_i: Colony Forming Unit at t=0h

II.8.3.2. Viability under simulated gastrointestinal conditions

The same procedure used with cells encapsulated in sodium alginate was followed. Viable cell counts were monitored during initial exposure to SGI conditions, after 2 h to assess the gastric transit tolerance and then after 4 h to evaluate the intestinal transit tolerance **(Vizoso *et al.*, 2006)**. It is calculated as follow:

$$\text{Viability (survival)} = \text{Log } CFU_t / \text{Log } CFU_i \times 100$$

CFU_t: Colony Forming Unit after the given period of storage

CFU_i: Colony Forming Unit at t=0h

II.9. Statistical analysis

Results are given as Mean±SD (Standard Deviation) of two independent replicates. Statistical comparisons of the results were determined by ANOVA followed by Tukey's, Sidak's or Dunnett's multiple comparisons tests using Graphpad Prism version 7.00 for windows; GraphPad Software, La Jolla California USA. Results were considered significant when $p<0.05$, $p<0.01$, $p<0.001$.

Results and discussion

This work aimed firstly to isolate and identify lactic acid bacteria (*Lactobacillus* spp) from a traditional cheese "Klila" having the best probiotic and technological traits and secondly to encapsulate them in different matrixes and follow their resistance to storage and gastrointestinal simulated conditions.

III.1. Isolation and identification

The presence of lactic acid bacteria (LAB) in traditional fermented products have been reported in several works, these bacteria include lactcocci, leuconostoc, and lactobacilli. In the beginning of this work, 40 colonies were picked out; however, only lactobacilli were selected to be studied later. 29 bacterial isolates were selected as lactobacilli and used for further studies. The colonies were first morphologically observed under light microscope. They were all rods, Gram-positive, and catalase negative and they were identified as *Lactobacilli* **(Pyar and Peh, 2014)**. ADH test, growth in NaCl concentration, and growth at different temperatures were also tested. A pre-identification was carried out based on carbohydrates fermentation, results were summarized in **tables 1 and 2 (appendixes)**.

According to the biochemical identification of **Hammes and Vogel, (1995)** and **Stiles and Holzapfel (1997)**, the isolates are principally belonged to the *Lb. plantarum* specie (62.06%), other species were also isolated such as *Lb. acidophilus* (17.24%), *Lb. helviticus* (6.89%), *Lb. casei* (6.89%), *Lb. fermentum* (3.44%) and *Lb. brevis* (3.44%). Our results indicate the predominance of the *Lactobacillus plantarum* compared to the total of lactobacilli. The biodiversity of LAB in Klila cheese observed in our study is in agreement of findings of **Boubekeri and Ohta (1996)**, where they showed that the microbial composition of Klila could change from region to region and comprises mainly *Lactobacillus*, *Enterococcus*, *Pediococcus* and *Leuconostoc*. In the study of **Guetouache *et al.* (2015a)** where lactic acid bacteria were isolated from butter, Klila and Jben, the authors reported that *Lactobacillus* specie varied from one cheese to another.

In another study of **Guetouache *et al.* (2015b)**, the lactic acid bacteria harboring the rural traditional cheese Jben of Djelfa province were found to be lactobacilli, lactococci and enterococci where the dominant lactobacilli were *Lb. plantarum, Lb. acidophilus, Lb. casei, Lb. fermentum* and *Lb. rhamnosus* and *Lb. helviticus* in different percentage. Our results also correlated with those of **Taboada *et al.* (2014)**, in their work, bacteria isolated from the traditional Argentinean goats milk products were *Lb. plantarum* (predominant), *Lb. fermentum, Lb. rhamnosus, Lb. casei, Lb. brevis, Lb. acidophilus* and *Lb. helviticus*. In

another work, **Guetouache and Guessas, (2015)** isolated 132 lactobacilli from Klila from Djelfa province; they reported the presence of *Lb. fermentum*, *Lb. plantarum*, *Lb. casei*, *Lb. brevis*, *Lb. acidophilus*, *Lb. intestinalis*, *Lb. helviticus* and *Lb. alimentarus*, corresponding to our findings. **Mechai *et al.* (2014)** also isolated *Lb. plantarum*, *Lb. brevis* and *Lb. acidophilus* from a variety of Algerian traditional fermented milk (Jben, Klila, Raib and Lben).

Molecular identification based on 16S rRNA technique for fourteen isolates was carried out. The results of the sequencing of the PCR product were compared with the GenBank database sequences using the BLAST program. 16S rRNA sequences of the isolates was deposited in GenBank with accession numbers listed in **table III.1**. The phylogenetic trees of the tow selected strains generate by Mega X program are presented in **figure III.1**.

Table III.1. Accession numbers of the isolated and identified LAB from Klila Cheese deposited in GenBank.

Isolate code	Strain	Accession number
Q1	*Lb. plantarum*	MH342639
Q2	*Lb. plantarum*	MH342637
Q3	*Lb. plantarum*	MH342635
Q5	*Lb. pentosus*	MH342629
Q6	*Lb. plantarum*	MH342628
Q8	*Lb. plantarum*	MH342631
Q9	*Lb. plantarum*	MH342636
Q14	*Lb. plantarum*	MH342630
Q18	*Lb. plantarum*	MH342626
Q20	*Lb. plantarum*	MH342638
Q28	*Lb. plantarum*	MH342627
Q30	*Lb. plantarum*	MH342632
B1	*Lb. casei*	KY764324
KBM2	*Lb. brevis*	KY764331

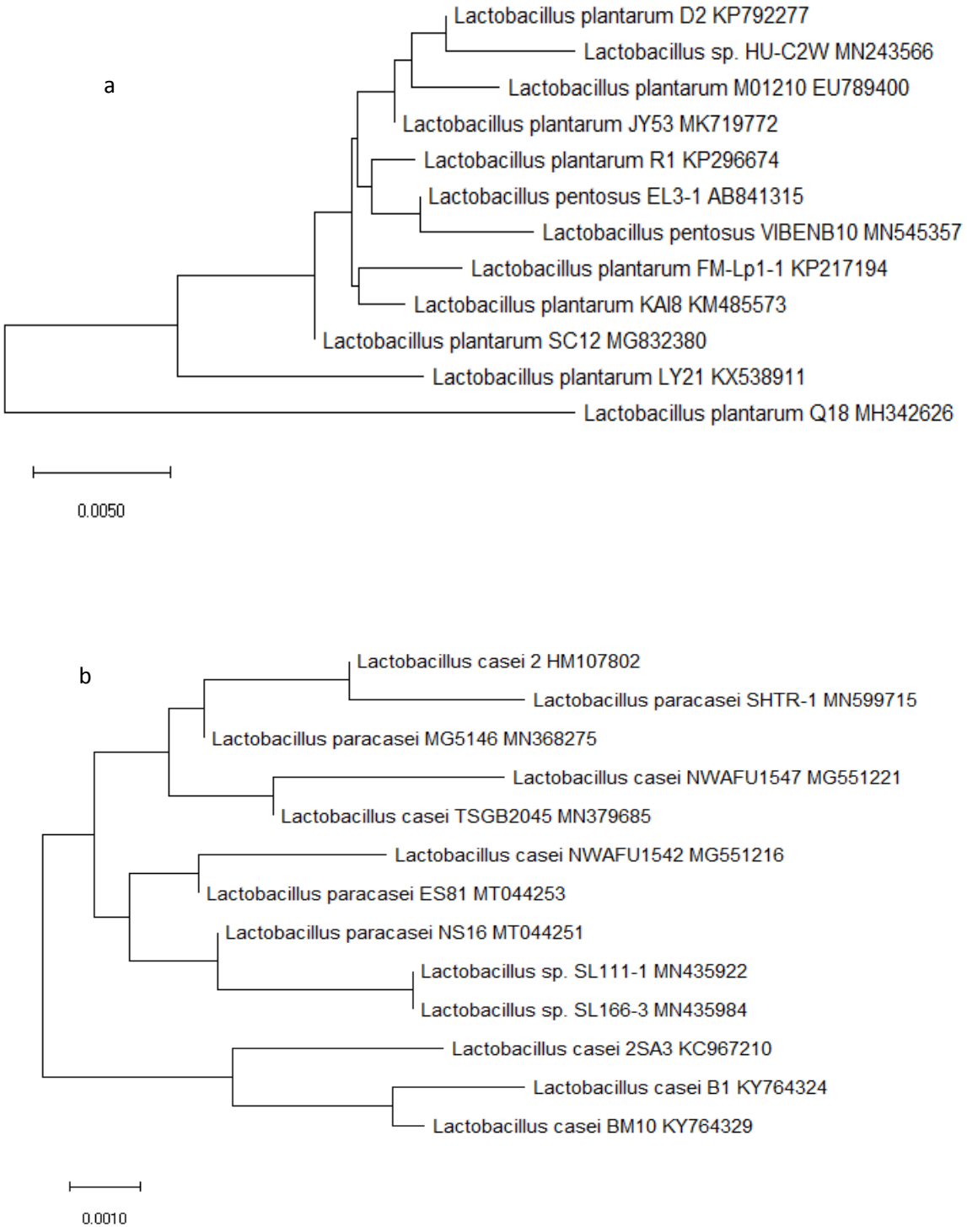


Figure. III.1. The phylogenetic trees of the tow selected strains generate by MEGA X program, (a) *Lb. plantarum* Q18 and (b) *Lb. casei* B1

III.2. Technological and probiotic properties

III.2.1. Technological properties

III.2.1.1. Acidifying ability

The acidifying function is the most sought after metabolic property of LAB used in the food industry. Variation of pH and the level of lactic acid produced expressed by Dornic degree in the skimmed milk inoculated with the twenty nine isolates were presented in **table III.2** and **table III.3**, respectively. The variation of acidification was monitored for all isolates**.** The decrease of pH of the milk is due to the production of lactic acid from lactose fermentation. The amount of lactic acid varies according to the isolates and their capacity and the rate of degradation of the lactose. Thus, according to the level of lactic acid produced, studied strains were classified into three groups: low (≤ 0.5% lactic acid), medium (between 0.5% and 0.7% lactic acid), and high (≥ 0.7% lactic acid) **(Taboada *et al.*, 2014)**.

As shown in **table III.2,** it is clear that change in pH is very slow after 3h of incubation, the change was found statistically significant with all isolates except Q28, Q8, K3, K7, and Q5. After 6 h of incubation it is noted that pH values were around 6.0 with high significant difference, ($p< 0.001$). For K1 and B1 where the pH decreased from 6.42±0.02 to 5.98±0.03 and from 6.40±0.04 to 5.91±0.01, respectively, it seems that these two isolates can be considered as fast acidifier. However, after 24h of incubation, pH reached the value of about 5.0 for the most of isolates, in addition, the only isolates which are able to reduce pH to 4 are Q18 (4.8±0.06) and B1 (4.49±0.01). The fast acidifying strains are therefore good candidates for dairy fermentation process as primary starter culture while the poor acidifier strains can be used as adjunct cultures depending on other properties **(Ayad *et al.*, 2004)**.

Table III.3 clearly showed the difference between isolates to metabolize lactose and produce lactic acid during 24h of incubation. Dornic acidity is expressed as degree Dornic (°D) **(1°D = 0.1 g lactic acid/liter)**. After 3h of incubation, the highest level of lactic acid produced corresponded to 32.5±0.71°D, was registered with B1 followed with Q9 with 32±1.41°D. After 6h of incubation, the highest values were 40±2.83 for Q5, 37.75±2.47 for K7, 41±4.24 for B1, while after 24h; the highest values were 95.5±4.95 for B1, 86.5±2.12 for KBM2 and 85±4.24 for Q18 with very high significant difference ($p< 0.001$) for all isolates compared to t0. Isolates with °D more than 50°D and less than 70°D were 4 isolates, K13, Q14, K11, and K29. Isolates with °D more than 70 were Q18, K17, B1, KBM2, K18. The remaining isolates were considered as low acidifying bacteria.

Table III.2. pH variation of different isolates incubated in skimmed milk (12%) for different time

Isolates	pH (t0)	pH (t1= 3h)	pH (t2= 6h)	pH (t3 =24 h)
Q28	6.49±0.08	6.425±0.11 ns	6.25 ±0.10***	5.335±0.04 ***
Q8	6.48±0.12	6.39±0.06 ns	6.235±0.05 ***	5.395±0.02 ***
Q1	6.49±0.01	6.35±0.07*	6.2±0.06 ***	5.415±0.02 ***
Q3	6.62±0.06	6.345±0.02 ***	6.24±0.07 ***	5.39±0.01 ***
Q9	6.48±0.01	6.305±0.08 **	6.19±0.08 ***	5.385±0.02 ***
Q20	6.54±0.04	6.265±0.08 ***	6.225±0.12 ***	5.8±0.06 ***
Q30	6.59±0.05	6.345±0.08 ***	6.23±0.07 ***	5.345±0.02 ***
Q18	6.46±0.01	6.245±0.09 ***	6.145±0.05 ***	4.8±0.06 ***
Q2	6.54±0.01	6.355±0.06 **	6.26±0.06 ***	5.395±0.02 ***
Q6	6.55±0.03	6.34±0.08 ***	6.25±0.08 ***	5.44±0.03 ***
K2	6.55±0.06	6.295±0.08 ***	6.24±0.08 ***	5.41±0.01 ***
K29	6.49±0.04	6.3±0.14 **	6.21±0.07 ***	5.325±0.06 ***
K17	6.51±0.03	6.335±0.08 **	6.19±0.10 ***	5.175±0.11 ***
K1	6.42±0.02	6.225±0.04 **	5.98±0.03 ***	5.205±0.02 ***
B1	6.40±0.04	6.16±0.01 ***	5.91±0.01 ***	4.49±0.01 ***
K3	6.35±0.06	6.25 ±0.04 ns	6.11±0.03 ***	5.315±0.11 ***
K4	6.39±0.06	6.245±0.02 *	6.085±0.02 ***	5.365±0.05 ***
K5	6.71±0.08	6.305±0.04 ***	6.28±0.03 ***	5.42±0.10 ***
KBM2	6.67±0.03	6.335±0.04 ***	6.25±0.06 ***	5.275±0.11 ***
K6	6.66±0.03	6.325±0.04 ***	6.29±0.03 ***	5.71±0.16 ***
K18	6.65±0.02	6.335±0.04 ***	6.265±0.02 ***	5.21±0.04 ***
K9	6.42±0.01	6.245±0.04 **	6.185±0.02 ***	5.385±0.05 ***
K10	6.40±0.01	6.22±0.03 **	6.085±0.05 ***	5.38±0.06 ***
K11	6.44±0.02	6.15±0.01 ***	6.1±0.03 ***	5.345±0.02 ***
K12	6.43±0.01	6.215±0.04 ***	6.125±0.02 ***	5.365±0.02 ***
K13	6.45±0.01	6.205±0.02 ***	6.125±0.04 ***	5.34±0.06 ***
Q14	6.49±0.01	6.315±0.04 **	6.195±0.02 ***	5.335±0.04 ***
K7	6.47±0.00	6.4±0.03ns	6.195±0.04 ***	5.44±0.04 ***
Q5	6.44±0.00	6.31±0.01 ns	6.18±0.03 ***	5.355±0.02 ***

Dunnett's multiple comparisons test was carried out to compare initial pH (t0) and pH at several times (t1, t2 and t3) for each bacterial strain. Results are expressed as Mean ± SD, (n=2). *$p < 0.05$,**$p < 0.01$,***$p< 0.001$, and ns (no significant difference) compared to t0.

Table III.3. Dornic acidity variation of different isolates incubated in skimmed milk (12%) for different time

Isolates	Dornic acidity (t0)	Dornic acidity (t1= 3h)	Dornic acidity (t2= 6h)	Dornic acidity (t3= 24h)
Q28	25.75±0.35	27.75±1.06 ns	29.5 ±0.71 ns	48.5 ±0.71 ***
Q8	27.5±0.71	28.5± 2.12 ns	28.75± 1.77 ns	47,75 ±0.35 ***
Q1	26.75±0.35	27.5 ±2.12 ns	28.75 ±1.06 ns	47.5± 2.12 ***
Q3	19.75±0.35	26± 1.41 **	28 ±1.41 ***	44 ±1.41 ***
Q9	30.5±0.71	32± 1.41 ns	28± 1.41 ns	43 ±1.41 **
Q20	21.25±1.06	29 ±1.41 ***	29.75± 0.35 ***	37 ±2.83 ***
Q30	23.5±0.71	26± 1.41 ns	28.5± 0.1*	49± 1.41 ***
Q18	29.5±0.71	30.5± 2.12 ns	36.5 ±0.71**	85± 4.24 ***
Q2	23±1.41	27.25±1.77 ns	27.5±0.71 ns	46± 1.41 ***
Q6	21.75±0.35	24.5±0.71 ns	27.75 ±1.06 **	45.5± 0.71 ***
K2	22.5±0.71	23.5± 0.71 ns	25.75 ±0.35 ns	43.25±1.77 ***
K29	24.5±0.71	25.5± 2.12 ns	34 ±1.41 ***	54.75±1.06 ***
K17	22.5±0.71	26.5±0.71 ns	35 ±1.41 ***	87 ±2.83 ***
K1	25.5±0.71	28.25±0.35 ns	31.75±1.06 **	46.75± 0.35 ***
B1	27.75±0.35	32.5±0.71 *	41 ±4.24 ***	95.5 ±4.95 ***
K3	27.5±0.71	29.5± 0.71 ns	31.25 ±1.06 ns	43.75± 0.35 ***
K4	27.5±0.71	30.25± 1.06 ns	33 ±1.41 *	42.5±2.12 ***
K5	18.25±1.06	21.5± 0.71ns	25.25 ±1.06 **	47.25 ±1.06 ***
KBM2	19.75±1.77	26 ±0.71**	23.5 ±2.12 ns	86.5 ±2.12 ***
K6	20.5±2.12	29± 1.41***	23.75±1.06 ns	42.5± 3.54 ***
K18	21.75±0.35	26.5±3.54 *	26.25±2.47 ns	83 ±2.83 ***
K9	27.25±2.47	25.75 ±1.06 ns	27.25± 1.77 ns	46.25±1.06 ***
K10	29±1.41	30 ±1.41 ns	35.25 ±3.18 **	44.5 ±2.12 ***
K11	29.5±0.71	31.25±1.77 ns	32.75± 0.35 ns	51± 0.71 ***
K12	29.25±1.06	29.75± 0.35 ns	32 ±1.41 ns	45.75 ±9.55 ***
K13	29.5±0.71	31.5± 2.12 ns	27.75±1.06 ns	53.5± 4.95 ***
Q14	26.5±0.71	21.5±3.54 *	30 ±1.41 ns	53 ±4.24 ***
K7	28.75±1.06	24.5 ±2.12 ns	37.75±2.47 ***	45.5±3.54 ***
Q5	30±0.71	31 ±1.41 ns	40± 2.83 ***	48.5±2.12 ***

Dunnett's multiple comparisons test was carried out to compare initial Dornic acidity (t0) and Dornic acidity at different times (t1, t2 and t3) for each bacterial strain. Results are expressed as Mean ± SD, (n=2). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ns (no significant difference) compared to t0.

It is mentioned that among lactic acid bacteria, *Lb. plantarum* is one of the most acidifying species; this is also a principal bacterium in the ripening step during cheese maturation **(Vescovo *et al.*, 1993; Todorov and Gombossi, 2010; Taboada *et al.*, 2014)**. Acidifying property is a very important criterion of LAB, in fact LAB having this ability are used as starter in the process of fermentation, furthermore, the low pH can considered as a mechanism by which LAB fight a large spectrum of pathogenic microorganisms, it can also, contribute in enhancement of the final products shelf life **(Ammor and Mayo, 2007)**.

The best acid producer was observed to be B1 which acidified the growth medium, thereby lowering the pH to 4.49 with 95.5±4.95°D followed by Q18 with pH of 4.8 and Dornic acidity 85±4.24°D. The amount of lactic acid produced by the isolates after 24h of incubation is ranging from 37°D to 100°D. This difference is related to the ability of the isolate itself to metabolize and ferment the existing compounds (**De Roissart, 1986)**. Moreover, variation in this aptitude may be related to difference in transport mechanism of sugars **(AL'benzino *et al.*, 2001)**.

III.2.1.2. Proteolytic ability

It is a feature that some lactobacilli have to provide amino acid requirements by hydrolyzing proteins. This aptitude is enzymatic equipment dependent. Lactobacilli are generally more proteolytic than lactococci **(Donkor *et al.*, 2007, Liu *et al.*, 2008, Iyer *et al.*, 2010)**. The 29 isolates were tested for their proteolytic ability and the obtained results showed a variation between isolates, some are proteolytic with a marked halo, while others are considered non proteolytic.

A low proteolytic activity was reported for LAB isolated from different origins (raw milk, fish and raw milk of Algerian dromedary) **(Ayhan *et al*., 2005; Thapa *et al*., 2006; Hassaine *et al*., 2007)**. Whereas, *Lb. plantarum* isolated from raw camel's milk didn't show proteolytic activity (**Edalati *et al.*, 2019)**. Another study of **Franciosi *et al.*, (2009)** reported that *Lc. lactis* subsp. *lactis*, *St. thermophilus*, *E. faecalis* and *E. durans* were proteolytic, but *Lb. plantarum* was not. However, results of **Guetouache *et al.* (2015a)** showed that strains *Lb. lactis* and *Lb. plantarum* isolated from Algerian dairy products had high proteolytic activity.

The proteolytic activity of dairy LAB is essential for the growth of the organisms in milk and it is involved in the development of organoleptic properties of different fermented products **(Christensen *et al*., 1999; Hassaine *et al*., 2007)**. **Wilkinson *et al.* (1994)** documented the

importance of the proteolytic activity of lactic acid bacteria because the texture of cheese is casein degradation dependent, this later is achieved with cell wall associated proteases and intracellular peptidases.

The results illustrated in **figure III.2** showed that only *Lb. casei* B1, *Lb. brevis* KBM2, *Lb. plantarum* Q18 and *Lb. plantarum* (K2) are proteolytic with diameters zones of (20.75±0.35) mm, (18.25±1.06) mm, (14.25±1.06) mm and (11.75±0.34) mm, respectively.

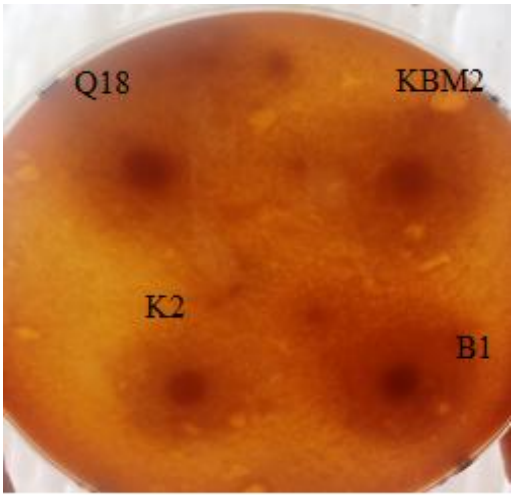


Figure.III.2. Proteolytic activity of the isolates *Lb. brevis* KBM2, *Lb. plantarum* Q18, *Lb. plantarum* (K2), *Lb. casei* B1 as shown on MRS agar supplemented with 10% skimmed milk.

III.2.1.3. Lipolytic ability

Negative results were obtained since none of the isolates had lipolytic property. This is not surprising knowing that lipolytic properties are generally low in LAB. Also, lipolytic activity of lactobacilli is generally lower than that of lactococci proving the weak lipolytic ability of *Lactobacillus* species as reported by **Papamanoli *et al.* (2003)**, however, they may be of interest for certain cheese applications. The flavor of the cheese is depending on the cheese variety and it is also due to the nature of the released fatty acids as result of action of lipolytic enzymes during milk fat hydrolysis **(Collins *et al.*, 2003)**.

III.2.1.4. Texturing ability

Many food grade microorganisms produce exopolysaccharides (EPS) **(De Vuyst and Degeest 1999, Sawadogo-Lingani, 2007)**, the ability of LAB to synthesize exopolysaccharides is important for the consistency and rheology of processed products and for improving the texture, and increasing viscosity of the finished products, they are considered as biothickeners **(De Vuyst and Degeest, 1999, Patel *et al.*, 2012)**. In our case, none of the isolates exhibited

this property. In the study of **Francios *et al.* (2009)**, none of the isolates showed this feature, while in the study of **Frau *et al.* (2016)**, four lactobacilli were able to produce EPS.

It was reported that EPS protect the producing microorganisms against dehydration and other harsh conditions such as acid and bile **(Fanning *et al.*, 2012; Weiner, 1995)**, and may also contribute to the aggregation properties required for colonization by probiotic lactic acid bacteria **(Walter et *al.*, 2008; Kojic *et al.*, 2011)**.

III.2.2. Probiotic properties

III.2.2.1. Antagonistic activity

Antagonism is one of the most important probiotic properties used to select LAB because it means they produce a variety of antimicrobial compounds that are used in the fermentation and bioconservation of food **(Mbawala *et al* ., 2013)**. These compounds include organic acids, such as lactic acid, acetic acid or propionic acid, hydrogen peroxide, carbon dioxide as a secondary metabolite, diacetyl, bacteriocins such as nisin, diplococcin, acidophilin, bulgarican **(Xie *et al.*, 2009; Ndagano *et al.*, 2011)**.The antimicrobial activity was determined by measuring the diameter of the inhibition zones around the spot using caliper in mm. The obtained results are presented in **table III.4.**

With respect to the antagonistic activity, the results obtained against the selected pathogenic bacteria, *S. aureus*, *L. monocytogenes*, *E. coli* ATCC 25922, *B. subtilus* and *Salmonella* sp. revealed that all isolates exhibited antagonist activity at least against one pathogen as in the case of K1, K17, K12 and K13, other isolates having antagonism activity against two pathogens such as Q8, K18, K4, K2, K3, K9, K7, K11, Q14 and Q5. Q1, Q3, K29, Q2 and Q6 had activity against three pathogens, whereas only Q9 had antagonistic activity against four pathogens. Q18 and B1 exhibited antagonistic activity against all tested strains.

Table III.4. Inhibition zones (mm) of lactic acid bacteria against the tested bacteria

	E. coli ATCC 25422	*Salmonella* sp.	*B. subtilis*	*S. aureus*	*L. monocytogens*
Q8	0±0 [a]	5.5±0.71	0±0 [a]	8±0 [b]	7.5±0.71 [b]
K18	0±0 [a]	7.5±0.71 [b]	0±0 [a]	0±0 [a]	7±0 [b]
Q1	6±0 [a]	7±1.41 [a b]	0±0 [c]	0±0 [c]	8±1.41 [b]
Q3	7.5±0.71 [a]	7±1.41 [a]	0±0 [b]	0±0 [b]	7.5±0.71 [a]
Q20	0±0.00 [a]	6.5±0.71 [b]	0±0 [a]	6.5±0.71 [b]	0±0.00 [a]
K1	0±0.00 [a]	0±0.00 [a]	0±0 [a]	0±0.00 [a]	8.5±0.71
K4	6.5±0.71 [a]	0±0.00 [b]	0±0 [b]	6.5±0.71 [a]	0±0.00 [b]
K2	4.5±0.71 [a]	0±0.00 [b]	0±0 [b]	5.5±0.71 [a]	0±0.00 [b]
K3	8.5±0.71	0±0.00 [a]	0±0 [a]	5.5±0.71	0±0.00 [a]
Q9	8.5±0.71 [a]	0±0.00	6.5±0.71 [b]	7.5±0.71 [a b]	7.5±0.71 [a b]
B1	10.5±0.71	7.5±0.71 [a]	6.5±0.71 [a]	6.5±0.71 [a]	12.5±0.71
K9	10.5±0.71	0±0.00 [a]	0±0.00 [a]	6±0.00	0±0.00 [a]
Q18	6.5±0.71 [a]	7.5±0.71 [a]	10.5±0.71 [b]	6.5±0.71 [a]	10±1.41 [b]
K29	7.5±0.71 [a b]	6.5±0.71 [a]	0±0 [c]	8.5±0.71 [b]	0±0 [c]
K5	7.5±0.71 [a]	0±0.00 [b]	0±0 [b]	6.5±0.71 [a]	0±0 [b]
Q28	8.5±0.71 [a]	6±1.41	0±0	9±0.00 [a]	12.5±0.71
KBM2	8.5±0.71 [a]	9.5±0.71 [a b]	0±0	11±1.41 [b]	10.5±0.71 [b]
K10	4.5±0.71	0±0.00 [a]	0±0 [a]	0±0.00 [a]	7±1.41
K6	8.5±0.71	0±0.00 [a]	0±0 [a]	6.5±0.71 [b]	6±1.41 [b]
Q30	8.5±0.71 [a]	0±0.00 [b]	0±0 [b]	10.5±0.71	8.5±0.71 [a]
Q2	7.5±0.71 [a]	0±0.00 [b]	0±0 [b]	10.5±0.71	6.5±0.71 [a]
K17	0±0.00 [a]	8.5±0.71	0±0 [a]	0±0.00 [a]	0±0.00 [a]
K12	0±0.00 [a]	0±0.00 [a]	0±0 [a]	11±1.41	0±0.00 [a]
K7	6.5±0.71 [a]	0±0.00 [b]	0±0 [b]	6.5±0.71 [a]	0±0.00 [b]
Q6	10.5±0.71 [a]	11.5±0.71 [a]	0±0 [b]	12±0.00 [a]	0±0.00 [b]
K11	8.5±0.71	0±0.00 [a]	0±0 [a]	11.5±0.71	0±0.00 [a]
K13	0±0.00 [a]	11.5±0.71	0±0 [a]	0±0 [a]	0±0.00 [a]
Q14	10.5±0.71	0±0.00 [a]	0±0 [a]	0±0 [a]	6.5±0.71
Q5	11.5±0.71	0±0 [a]	0±0 [a]	0±0 [a]	6.5±0.71

The effects of pathogenic bacteria on each bacterial species were compared. Diameters of inhibition (mm) were presented as Mean±SD; (n=2). Tukey's multiple comparisons test was used, results were considered significant when $p<0.05$. Within each raw, cells labeled with the same letters present no significant difference.

However, the results indicated that the degrees of antagonistic activity exhibited by LAB varied against the tested strains. The largest diameter zone was noted with B1 against *L. monocytogens* with an inhibition zone diameter of 12.5±0.71 mm, the same isolate gave a diameter zone of 10.5±0.71 mm against *E. coli* ATCC 25922 with significant difference, however, antagonism toward *Salmonella*, *Bacillus* and *Staphyloccocus* showed no significant difference with the following values 7.5±0.71 mm, and 6.5±0.71 mm. It is important to mention values of Q14 and Q5 against *E. coli* since diameters were 10.5±0.71 and 11.5±0.71 mm, respectively with significant difference ($p<0.05$). These results indicated that *E. coli* ATCC 25422 and *S. aureus* were more sensitive to antimicrobial agents produced by the examined isolates, compared to the other indicators.

The same results were reported by **Azzizi *et al.* (2017).** According to **Akabanda *et al.* (2014)** weak inhibition, moderate inhibition, and strong inhibition were considered when the diameter is less than 1-4 mm, better than 4-8 mm, and better than 8-12 mm, respectively, and based on these ranges, our isolates showed strong inhibition against *E. coli* 25422 (11 isolates), *S. aureus* (8 isolates) and *L. monocytogenes* (5 isolates), however, no inhibition was observed with 26 isolates in the case of *B. subtilis*. Our results are in agreement with those of **Guetouache and Guessas (2015)**, where *Lb. plantarum* and *Lb. brevis* isolated from Klila had antagonist activity toward *S. aureus* with diameters of 6.05 and 9.27 mm, respectively. In the study of **Guetouache *et al.* (2015b)**, the isolates, *Lb. plantarum*, *Lb. acidophilus* differ from weak to strong, and *Lb. casei* showed a strong antagonist activity and antimicrobial activity was observed towards *L. monocytogenes* and *S. aureus* but not *E. coli* and *Salmonella enteritidis*.

Furthermore, the obtained results are also in agreement with those of **Owusu-Kwartenz *et al.* (2015)** within *Lactobacillus* strains (*Lb. fermentum*) exhibited antagonism against pathogenic microorganisms *S. aureus*, and *L. monocytogenes*. The profiles of antagonism activity of LAB regarding pathogenic strains have been documented in many publications, results differ from strain to another, for instance, in the study of **Jans *et al.* (2012)** diameters were 4.2 mm for *E. coli*, 4.3 mm for *S. typhimurium* and 5.0 mm for *L. monocytogenes*.

Different Gram positive and Gram negative strains have been found sensitive to a variety of LAB. In fact, the inhibitory effect of *Lb. plantarum* toward Gram negative bacteria strains was reported **(Messi *et al.*, 2001)**. In addition, the study of **Mechai *et al.* (2014)** reported that their strains highly inhibited the growth of *L. monocytogens* and *S. aureus.* The study of

Haghshenas *et al.* (2017) showed that the fermented dairy products are rich in LAB with effective potential against pathogens; *Lb. plantarum* 15HN, showed diameters of 11.7, 13.7, 12.3, and 12.3 mm toward *S. aureus*, *L. monocytogenes*, *S. typimurium*, and *E. coli*, respectively. An efficient bacterium *(Lb. casei* TN-2A) with remarkable antimicrobial activity toward *E. coli* and *S. aureus* was also isolated from fermented camel milk **(Lü X *et al.*, 2014)**. In addition, the *Lb. acidophilus* AA105 strain isolated from raw camel milk strongly inhibited *Staphylococcus* sp., *Bacillus* sp., *S. paratyphi*, *Shigella* sp. and *E. coli* **(Abo-Amer, 2013)**. The study of **Bassyouni *et al.* (2012)** demonstrated *Lactobacillus* bacteria isolated from Egyptian dairy product showed a high antagonism effect against *E. coli* and *S. typhimurium* with an inhibition zones between 17 and 21mm.

Our results showed that the antagonist activity is species dependent; this observation is in agreement with previous reports **(Angmo *et al.*, 2016; Abushelaibi *et al.*, 2017)**. Positive antagonistic activity was also reported by **Weldesiet *et al.* (2019)** where *Lactobacillus plantarum* strain CIP 103151 gave values between 19.33 and 21 mm against the pathogens: *S. aureus*, *L. monocytogenes*, *E. coli*, and *S. typhimurium.* Moreover, the study of **Tigu *et al.* (2016)** showed the existence of antagonism activity of LAB from traditional Ethiopian fermented products toward *S. typimurium* and *E. coli* with inhibition zones ranged from 10.3 to 14.3 mm.

III.2.2.2 Antibiotic Resistance

Lactic acid bacteria are naturally resistant to many antibiotics due to their structure and physiology. The choice of the five tested antibiotics was based on the discrimination between intrinsic and acquired resistance. Results of antibiotic susceptibility are presented in **table III.5**. In general, for safety reasons, the absence of antibiotic resistance in LAB to be used as starter cultures or co-cultures is of great importance.

Table III.5 showed the response of the 29 tested LAB strains to antibiotics of different groups; cell wall inhibitors (Penicillin G, Amoxicillin) and protein synthesis inhibitors (Streptomycin, Gentamycin), and Colistin sulfate. The isolates were considered as susceptible, S (≥21 mm); intermediate, I (16-20 mm) and resistant R (≤15).

Table III.5. Susceptibility of lactic acid bacteria towards some antibiotics (diameters in mm)

	Penicillin G (10 µg)	Amoxicillin (25 µg)	Colistin sulfate (50 µg)	Gentamycin (10 µg)	Streptomycin (30 µg)
K1	16.75±0.35	21.5±0.71 [a]	23.5±0.71 [a b]	26±1.41 [b]	24±1.41 [a b]
K2	18±1.41	22.25±0.35 [a]	22.5±0.71 [a]	24.25±1.06 [a]	24.5±0.71 [a]
K3	12±1.41	24.5±0.71 [a]	24.25±1.06 [a]	25.75±1.77 [a]	24.5±0.71 [a]
K4	17.25±0.35	21.5±0.71 [a]	25.5±0.71 [b]	24.5±0.71 [b]	24±1.41 [a b]
K5	17.75±0.35	21.5±0.71 [a]	23.5±0.71 [a b]	26±1.41 [b]	24±1.41 [a b]
K6	18.5±0.71	25.25±1.77 [a]	24.75±1.06 [a]	22.5±1.41 [a]	24.75±1.06 [a]
K7	18±0.71	22.5±0.71 [a]	24.25±1.06 [a]	24.25±1.06 [a]	22±0.71 [a]
Q18	17.25±0.35	24.25±1.06 [a]	9±1.41	22±0.71 [a]	24±0.00 [a]
K9	18.75±0.35 [a]	24.5±0.71 [b]	16.75±1.06 [a]	23.75±0.35 [b]	22±0.00 [b]
K10	18±1.41	25.25±1.06 [a]	23.25±1.06 [a]	23±1.41 [a]	13.25±0.35
K11	18.75±0.35	24.25±1.06 [a]	24±1.41 [a]	24.7±0.42 [a]	24.5±2.12 [a]
K12	9.75±1.06	24.25±1.06 [a]	22.5±0.71 [a]	22.5±2.12 [a]	24±1.41 [a]
K13	16.75±0.35	25.25±1.06 [a]	24.5±0.71 [a]	23.75±0.35 [a]	26.5±2.12 [a]
Q14	18.5±0.7 [a]	17±0.00 [a]	25±1.41 [b]	25±0.71 [b]	25.75±0.35 [b]
B1	20±0.00 [a]	18.75±1.06 [a]	24.75±1.06 [a]	11.5±0.71	23±0.00 [b]
Q5	11.25±1.06	24.5±0.71 [a]	24.25±1.06 [a b]	26±1.41 [a]	21.5±0.71 [b]
K17	18.75±0.35 [a]	22.75±0.35	9±1.41	25.75±1.06 [b]	23±1.41 [a b]
K18	18±1.41	23.5±0.71 [a b]	21.5±0.71 [b]	26±2.83	23.25±0.35 [a b]
KBM2	11.5±0.00	25.25±0.35 [a]	22.75±0.35 [a b]	23±1.41 [a b]	22.25±0.35 [b]
Q28	17.75±1.77	25.25±1.06 [a b]	7.5±0.71	27.5±2.12 [a]	24.25±0.35 [b]
Q8	16.75±0.35	23.25±1.06 [a]	6±0.00	26±1.41 [a]	24±1.41 [a]
Q1	23±1.41 [a]	25.75±0.35 [a b]	11.25±0.35	27±1.41 [b]	23.75±1.77 [a]
Q3	17±0.00	24.5±0.71 [a]	8.5±0.71	24.25±1.06 [a]	24.75±1.77 [a]
Q9	19.5±0.71 [a]	26.75±0.35 [a]	11±1.41	25±1.41 [a]	22±0.00 [a]
Q20	19.25±1.06	23.75±0.35 [a]	8±1.41	23±1.41 [a]	24.25±1.77 [a]
Q30	17.5±0.71	26±1.41	7.5±0.71	23±1.41 [b]	25±0.00 [a b]
Q2	19±1.41	22.75±0.35 [a]	8.5±0.71	23.5±0.71 [a]	24.75±1.77 [a]
Q6	18±1.41	24±0.00 [a]	10±0.00	24.5±2.12 [a]	24.75±1.06 [a]
K29	18.25±0.35	25±0.00 [a]	11.5±0.71	23.25±1.06 [a]	23±0.71 [a]

Zones of inhibition (diameter in mm) for each antibiotic are given as Mean±SD. The effects of antibiotics on each bacterial species were compared. Tukey's multiple comparisons test was used. Results were considered significant when $p<0.05$. Within each raw, cells labeled with the same letters present no significant difference.

The majority of our isolates are sensitive to the 5 antibiotics used with near diameters while the largest one was 27.5±2.12 mm obtained with Q28 with Gentamycin and the smallest one was 6 mm obtained with Q8 with Colistin sulfate. All isolates were Amoxicillin (except Q14) and Gentamycin (except B1) sensitive, K10 is the only resistant isolate to Streptomycin with a diameter of 13.25±0.35mm, and 12 isolates are resistant to Colistin sulfate (Q18, K17, Q28, Q8, Q1, Q3, Q9, Q20, Q30, Q2, Q6, K29), while intermediate results were recorded with Penicillin. In terms of significance between the different antibiotics regarding each bacterial species ($p<0.05$), results showed that in some cases there is no significant difference in diameters of all the antibiotics used with all isolates: this is the case of K9, Q14, K18, KBM2 for example, however the remarkable significance ($p<0.05$) was found with Gentamycin compared to the other antibiotics, as example K4 (24.5±0.71), K5 (26 ±1.41 mm).

Variable results of resistance of LAB toward antibiotics were documented, for example **Gad (2014)** showed that LAB isolated from food products were still sensitive to Gentamycin and Erythromycin. It has also been reported that strains of *Lb. paracasei subsp. paracase*i were resistant to aminoglycosides (Gentamycin, Kanamycin) and to Tetracycline **(Charteris *et al.*, 1998; Zhou *et al.*, 2005; Ammor *et al.*, 2008; Ripamonti *et al.*, 2011)**.

The work of **Davidson *et al.* (2019)** showed that almost of their isolates were sensitive to Ampicillin and Amoxicillin, against only 5.9% of *Lb. bulgaricus* isolates showed susceptibility to Streptomycin, Neomycin, or Gentamycin. Moreover, resistance of LAB isolated from another traditional fermented product (Dahi) was also documented, where all strains of *Lb. rhamnosus* were sensitive regarding the tested antibiotics **(Ramachandra *et al.*, 2009)**. Nevertheless, the study of **Francios *et al.* (2009)** showed that lot of LAB strains were resistant to the tested antibiotics. In some studies, isolated lactobacilli (fermented food, cheese, human origin) were susceptible to inhibitors of cell wall synthesis, such as Ampicillin (**Mandar *et al.*, 2001**; **Belletti *et al.*, 2009)** while in other studies they were resistant to Penicillin (**Temmerman *et al.*, 2003; Flórez *et al.*, 2005**; **Machiour *et al.*, 2016)**, moreover, **(Danielsen and Wind (2003)** reported the natural resistance to Cefoxitin, Ciprofloxacin, Norfloxacin, Gentamycin, Sulfamides/Trimethoprim of their isolates.

Wide discussions about resistance and susceptibility of probiotic strains to antibiotics appeared, the problem is concerning the selection of resistant bacterium as probiotic. In fact, antibiotic resistance can be divided into two types intrinsic and acquired. Intrinsic antibiotic resistance is native (natural), encoded in chromosome cannot be inherited to other species.

There is some composition in the cell membrane that makes the bacterium resistant. The second one (the acquired resistance) is plasmid encoded and transferrable between species, hence, occurred the natural intestinal microbiota and gene transfer **(Danielsen and Wind, 2003; Davidson, 2019)**.

Resistance to aminoglycoside antibiotics, such as Gentamycin, Streptomycin and Kanamycin, is considered to be intrinsic and so resistant genes are carried on the chromosome in the *Lactobacillus* genus and it is attributed to the absence of cytochrome-mediated electron transport, which mediates drug uptake (**Danielsen and Wind, 2003)**. Acquired resistance of LAB to antibiotics is not preferred, because genes may be transferred to pathogenic bacteria and so render them resistant, however, it may have advantage or good effect in the case of intake of resistant probiotics and antibiotics.

III.2.2.3. Resistance to acid pH

The **figure III.3** showed the survival of the isolated bacteria in acidic pH (pH=03). It is clear that the 29 isolates survived differently in low pH. *i.e*, the best resistance was 81.3%, it was observed with B1, and 79.4% with Q18 and 77.1% with KBM2, Q8 the survival was 69.7%, Q30 (70.3%), 67.5% with Q6, 66.07% with Q9, 65.3% with Q20, and it was 52.8% with K2, the remaining isolates showed survival less than 50%.

Resistance of LAB to low pH was reported in previous studies (**Xanthopoulos *et al.*, 2000; Park *et al.*, 2002; Maragkoudakis *et al.*, 2006; Ramos *et al.*, 2013)**. More than 80% survival was noted with *Lb. plantarum* strains at pH 2 for 3h incubation in findings of **Akalu *et al.* (2017)** and **Rajok *et al.* (2017)**. Over 56 isolates, 17 LAB were also reported resistant and survived in low pH (2 and 3) for 3h (**Weldesiet *et al.* 2019)**. Four (04) *Lb. plantarum* species isolated from fermented olives resisted pH 2 during 2h of incubation, the least survival was 49%, while the highest was 65% **(Mourad and Nour-Eddine, 2006)**. **Jacobsen *et al.* (1999)** found that only 29 isolates were considered as resistant to pH 2.5 for a period of 4 hours from an initial number of 44 isolates. Also, **Owusu-Kwartenz *et al.* (2015)** reported the resistance of 16 isolates from a total of 48 strains of *Lb. fermentum* exposed to pH 2.5 for 4h.

Viability of LAB in low pH is a principal probiotic aptitude, the acidic pH was found to inhibit growth and metabolism of bacteria **(Sultana *et al.*, 2000; Chan and Zhang, 2005)**. Viability is desired in stomach like conditions wherein, pH is approximately two. So a resistant bacterium in such medium is able to reach gut and exert the beneficial effects contrary to bacteria with low resistance.

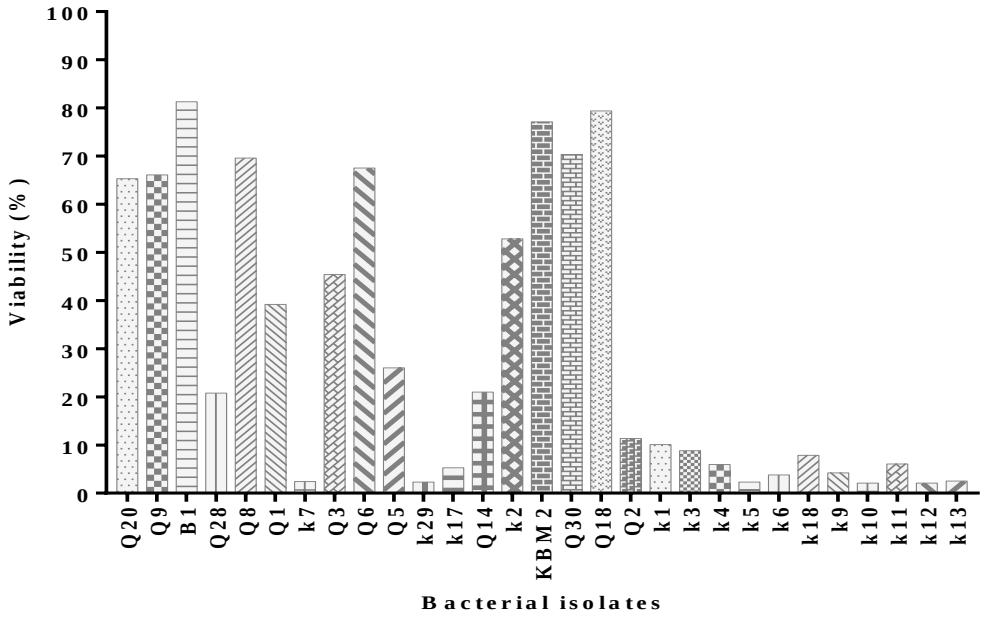


Figure III.3. Viability of isolated bacteria in acidic pH (pH 3) after 2h of incubation.

III.2.2.4. Resistance to gastrointestinal conditions

The survival of bacteria in gastric juice depends on their ability to resist the low pH and the enzymatic action, also, in simulated intestinal juice, probiotic bacteria must survive in these hostile conditions in order to achieve colon. Fourteen (14) genetically identified isolates were screened for their resistance to gastrointestinal like conditions: gastric simulated juice (GSJ) (pH 2.5, for 2h) and intestinal simulated juice (ISJ) (pH 7.5, for 2h) and viability was tested and presented in **figure III.4**; as demonstrated, the viability of the fourteen isolates during gastric simulated juice, it is clear that in gastric simulated conditions, survival of six (06) isolates was more than 50%, they were B1 (80.98 ±1.24), KBM2 (86.11±8.48)%, Q18 (81.37±19.97) %, Q6 (76.99±14.59)%, Q8 (66.64± 0.97) %, Q9 (56.16)%.

The second barrier which probiotic bacteria must pass is intestinal barrier; therefore, the fourteen identified bacteria were also tested for their intestinal resistance. In intestinal simulated conditions, isolates with viability more than 50% were, Q18 (79.41±2.77)%, KBM2 (76.61±7.41)%, Q6 (68.76±1.59)%, Q8(61.32±7.57)%, and B1 (54.62±6.26)%. The above

results showed that viability is strain dependent. This property was reported in different works **(Maragkoudakis *et al.*, 2006; Argyri *et al.*, 2013; Owusu-Kwartenz *et al.*, 2014; Bouridane *et al.*, 2016)**.

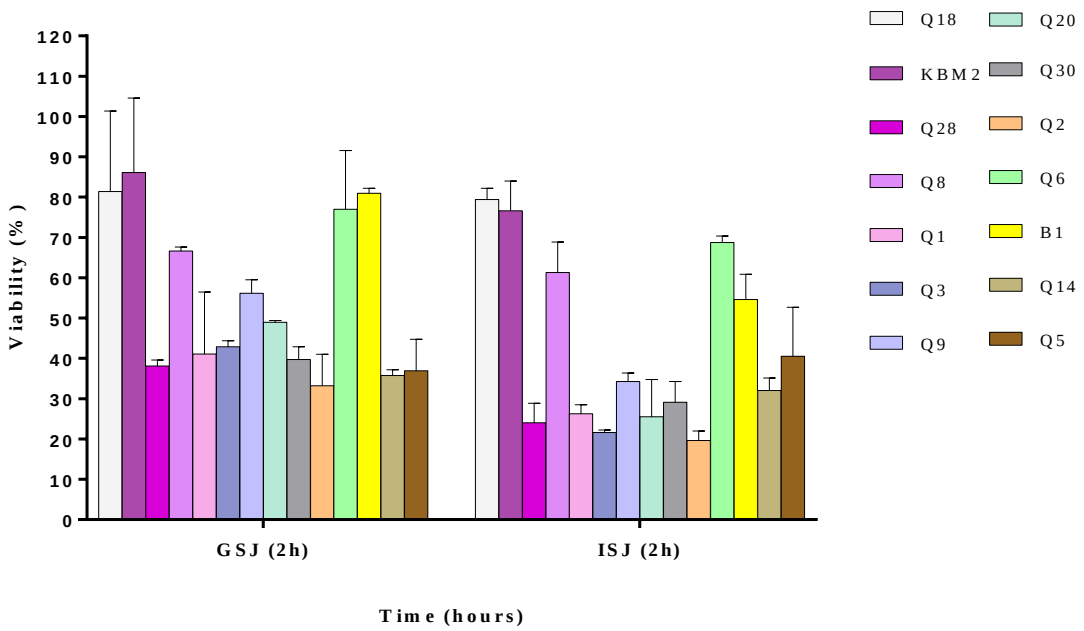


Figure III.4. Viability of isolates in simulated gastric juice (pH2.5, 2h) and in intestinal simulated juice (pH 7.5, 2h).

Based on the previous results of technological and probiotic properties, two isolates, *Lb. casei* B1, and *Lb. plantarum* Q18 were selected and were subjected to the resistance to bile salts, autoaggregation, hydrophobicity, adhesion and hemolysis.

III.2.2.5. Resistance to bile salts

For the resistance to bile salts and as indicated in **table III.5**, the two isolates were resistant and the recorded survival rate was 83.78% for *Lb. casei* B1 and 75.53% for *Lb. plantarum* Q18. In findings of **Argyri *et al.* (2014)**, most of the isolated *Lb. plantarum* and *Lb. pentosus* had high resistance to bile salts. The importance of testing resistance to bile salts come from the effect that they disrupt bacterial homeostasis and cause cell death as a result of the detergent property they have **(Sahadeva *et al.*, 2011)**. It was reported that resistance to bile salts is strain dependant **(Charteris *et al.*, 1998; Xanthopoulos *et al.*, 2000)**.

III.2.2.6. Hemolytic activity

The general absence of hemolysis or poor hemolytic activities expressed by lactic acid bacteria is indicative of their safety applications in food. This proves the nonpathogenic status

of the probiotic isolates. *Lb. plantarum* Q18 and *Lb. casei* B1 are not hemolytic since"no zone" appeared in the plates inoculated with the studied isolates. The study of **Owusu-Kwartenz *et al.* (2015)** showed that all isolates were safe since none of the tested *Lb. fermentum* strains showed β- haemolytic activity. *Lactobacillus* sp. isolated from dairy products **(Maragkoudakis *et al.*, 2006)**, fermented olives (**Argyri *et al.*, 2013)** and different African fermented food products **(Adimpong *et al.*, 2012)** also exhibited safety pattern.

III.2.2.7. Adhesion to epithelial cells, hydrophobicity and autoagregation

Adhesion is a criterion of paramount importance because it is a condition for colonization of the intestines. In addition to the adhesion to the epithelial cells of the intestine, probiotics can attach to the mucus that covers the enterocytes or to the various microorganisms found in the gastrointestinal tract **(Ingber *et al.*, 2000)**. Adhesion of *Lb. plantarum* Q18 and *Lb. casei* B1 to intestinal epithelial cells was presented in **figure III.5**. It was good for *Lb. plantarum* Q18 and for *Lb. casei* B1 too.

The beneficial effect of adhesive probiotic bacteria is mainly due to competition and elimination of pathogens to adhere to intestine surfaces. Bacterial cell-surface proteins associated with mucus and intestinal cells are the responsible agents of this property **(Veléz *et al.*, 2007; Sánchez *et al.*, 2008)**. It seems that the attachment of *Lactobacillus* strains to intestinal cells is mediated by cell surface components and extra-cellular factors **(Araki and Ito, 1989)**.

Our isolates were also tested for their cell surface hydrophobicity using the hydrocarbon xylene. They showed good hydrophobicity with levels of 65.21 % and 58.33 % for *Lb. casei* B1 and *Lb. plantarum* Q18, respectively. These levels were lower than values found in the study of **Sharma *et al.* (2016)** which showed a hydrophobicity of 93% for *Lb. casei* toward xylene. The study of **Somashekaraiah *et al.* (2019)** showed that hydrophobicity differed between isolates; some showed high levels of hydrophobicity (77.82%, 71.59% with MYSN 106 and MYSN 98, respectively) while others exhibited low levels (51.10% with MYSN 43). The aggregation level was between 50.29% and 78.95% and adhesion ability was with 50-100 bacterial cells per epithelial cell. In the study of **Todorov *et al.* (2011)**, the reported hydrophobicity was 68.7 % for *Lb. plantarum* ST16Pa. It was reported that adhesion of lactic acid bacteria to the membrane of enterocytes may be enhanced if bacteria are more hydrophobe **(Todorov *et al.*, 2009)**. However, **Bouridane *et al.* (2016)** showed that their isolates had low hydrophobicity and adhesion varied from an isolate to another.

Autoaggregation is the bacterial aggregation between cells of the same strain; it is an essential mechanism for biofilms formation. This characteristic is the result of interaction between the cells surface molecules (proteins and exopolysaccharides, also calls as autoagglutinins) **(Trunk *et al.*, 2018)**. Our isolates showed autoaggregation of 79.33 % and 72.66 % for *Lb. casei* B1 and *Lb. plantarum* Q18, respectively.

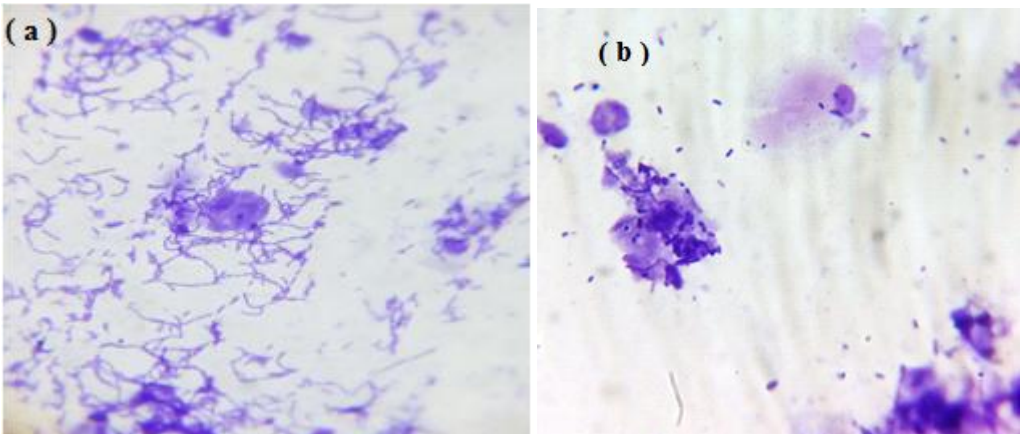


Figure III.5. Adhesion of (a) *Lb. plantarum* Q18 and (b) *Lb. casei* B1 to poultry ileum epithelial cells observed with optical microscope (x100).

Table III.6. Autoaggregation, hydrophobicity, hemolysis and bile salts resistance of *Lb. plantarum* Q18 and *Lb. casei* B1.

	Hydrophobicity %	Autoaggregation %	Hemolysis	Bile salts tolerance %
Lb. casei B1	65.21	79.33	-	83.78
Lb.plantarum Q18	58.33	72.66	-	75.53

(-) : absence of hemolysis

III.3. Encapsulation of *Lb.casei* B1 and *Lb. plantarum* Q18

III.3.1. Encapsulation of *Lb. casei* B1and *Lb. plantarum* Q18 with sodium alginate

III.3.1.1. Tolerance of free and encapsulated bacterial cells to acid stress

The effect of pH on the viability of free and encapsulated bacteria was tested at different times of incubation and at three different pH values (2, 4 and 7). The results presented in **figures III.6** and **figure III.7** for *Lb. casei* B1 and *Lb. plantarum* Q18, respectively, showed that the lowest viability was recorded at pH 2 and it decreased as the time of storage increased. For example, the number of *Lb. casei* B1 free cells was reduced by 8 Log CFU/ml after 7 days and by 9 Log CFU/ml after 14 days, however, encapsulated ones were reduced only by 2 Log CFU/ml after 7 days and by 2.8 Log CFU/ml after 14 days with high significant difference between free and encapsulated cells ($p < 0.01$).

For *Lb. plantarum* Q18, the cell number decreased by 6.9 Log CFU/ml after 7 days and by 9 Log CFU/ml after 14 days for free cells and by 2.3 Log CFU/ml and 2.6 Log CFU/ml after 7 and 14 days, respectively for encapsulated cells with very high significant difference ($p < 0.001$). The optimal pH for storage for both isolates and in both cases (free or encapsulated) was 7.

The study of **Bosnea *et al.* (2014)** reported similar results**,** since at low pH values; the viability of free cells decreased significantly, however, encapsulated ones showed a higher survival rate suggesting that sodium alginate microenvironment is offering to cells an acid resistance feature. In contrast, and after 3 h of incubation at 4°C, no effect was observed on the viability of both free and encapsulated forms, at pH 4.0 and pH 7.0. In a report of **Krasaekoopt *et al.* (2006)**, microencapsulated *Lb. acidophilus* and *Lb. casei* in sodium alginate and chitosan beads were added to yoghurt at pH 4.7 and stored at 4°C. Viability increased by one cycle Log compared to free cells in the cited conditions.

According to **Bosnea *et al.* (2014)**, the restored viability of bacterial cells at low pH environments, is attributed to the presence of membrane proton pumps or proton/cation exchange systems, their role is to maintain the cytoplasm pH near neutrality by controlling the influx of protons. In highly acidic pH conditions (H^+ concentration is very high), cells will be disrupted and will consequently lose their viability, this is actually due to the intracellular acidification resulting from the drastic decrease in pH gradient (the difference between the intracellular and the extracellular pH), caused by the dysfunction of the pH regulatory pumps.

Alginate microencapsulation is providing an additional physical defense mechanism to the probiotic cells through a barrier effect.

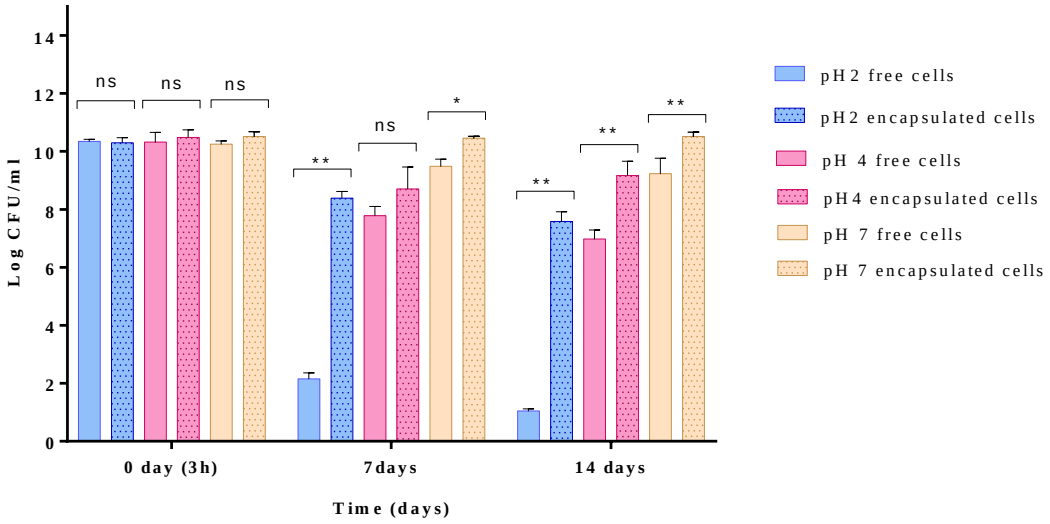


Figure III.6. Viability of free and encapsulated *Lb. casei* B1 at different pH values (pH=2, pH=4, pH=7) after their storage at 4°C for 3 h, 07 and 14 days. Results are expressed as Mean±SD, (n=2). Sidak's test was used. *p < 0.05, **p < 0.01, and ns (no significant difference) compared to free cells.

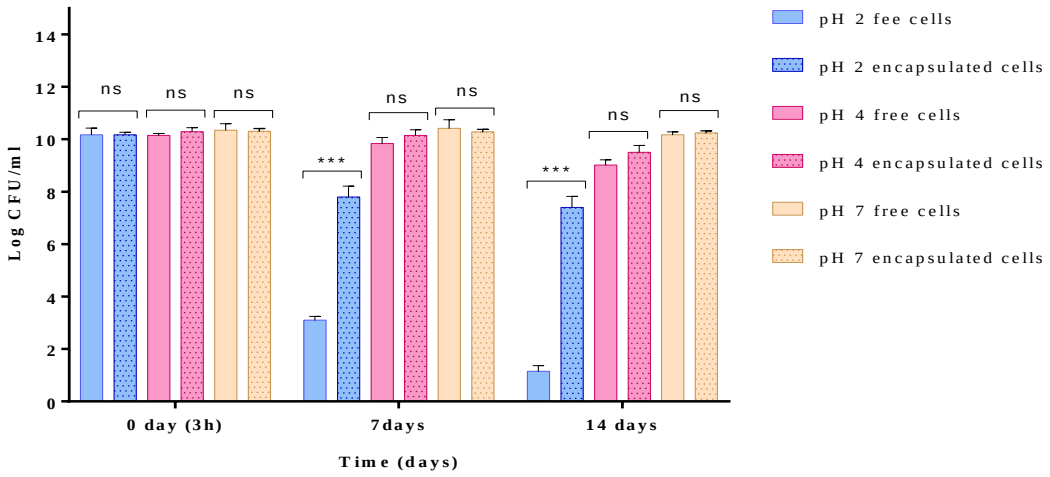


Figure III.7. Viability of free and encapsulated *Lb. plantarum* Q18 at different pH values (pH=2, pH=4, pH=7) after their storage at 4°C for 3 h, 07 and 14 days. Results are expressed as Mean ± SD, (n=2). Sidak's test was used. ***p < 0.001, and ns (no significant difference) compared to free cells.

III.3.1.2. Tolerance to NaCl

The effect of different salt (NaCl) concentrations on the survival of free and encapsulated cells is presented in figure **III.8 (a, b, c)** for *Lb. casei* B1 and in figure **III.9 (a, b, c)** for *Lb. plantarum* Q18. As clearly shown in the results, the number of encapsulated viable cells was higher than that of free ones at all salt concentrations, it remained unchangeable. Moreover, viability was better at the concentration of 3% compared to the other concentrations (6% and 9%) for *Lb. casei* B1 free cells, where at 3% it was reduced by 1.7 Log CFU/ml after 28 days of storage and by 4 Log CFU/ml and 9 Log CFU/ml after 14 and 28 days, respectively. At 6%, it was reduced by 8.06 Log after 21 days and 9.12 cycles after 28 days and at 9%, it was reduced by 3.6 Log CFU/ml and 9 Log CFU/ml after 7 and 14 days, respectively with very high significance between free and encapsulated cells ($p < 0.001$).

Lb. plantarum Q18 free cells were able to resist both 3% and 6% NaCl, but at 9%, viability was reduced by 9 Log CFU/ml after 14 days of storage at 4°C. However, encapsulated cells for both isolates resisted all salt concentrations. These results indicated that these bacteria resist harsh conditions of osmolarity, and showed that sodium alginate gives more protection to bacterial cells to resist such conditions of stress. In addition, the concentration of 3% gave the highest resistance and viability, but it decreased by the increase of salt concentration. Our results agree with those of **Gomes *et al.* (1998)** where they reported that the number of *Lb. acidophilus* decreased by the increase of salt concentration above 3.0%. Furthermore, in a study of **Cruz *et al.* (2015)**, when free *Lb. acidophilus* cells were exposed to 6% NaCl, viability decreased and by consequence, the microorganisms become less resistant to osmotic stress. The observed decline in the survival and resistance during storage could be attributed to the reduction in water activity and to the increase in osmolarity **(Jorgensen *et al.*, 1994)**.

Salts are usually added to foods as a taste enhancer or to prevent spoilage, therefore, at higher levels, the NaCl content could negatively influence viability of the probiotic cells. However, little is known on the mechanism by which microencapsulated probiotics resist to high salt concentrations found in cheese and other salted foods **(Bosnea *et al.*, 2014)**.

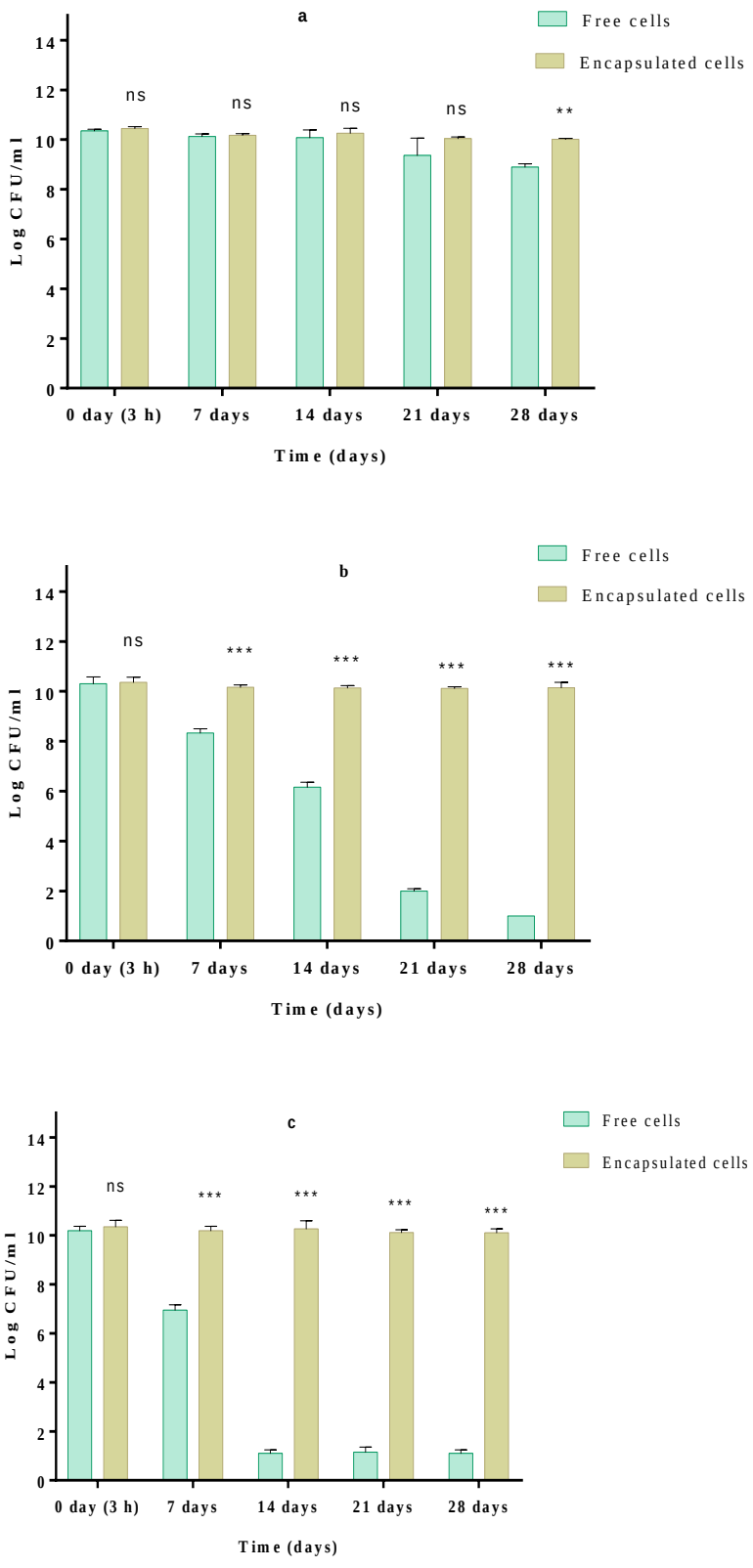


Figure III.8. Viability of free and encapsulated *Lb. casei* B1 at different NaCl concentrations (3%) (a), 6% (b), 9% (c)) after their storage at 4°C for, 3h, 07, 14, 21 and 28 days. Results are expressed as Mean ± SD, (n=2). Sidak's test was used, **p <0.01, ***p <0.001, and ns (no significant difference) compared to free cells.

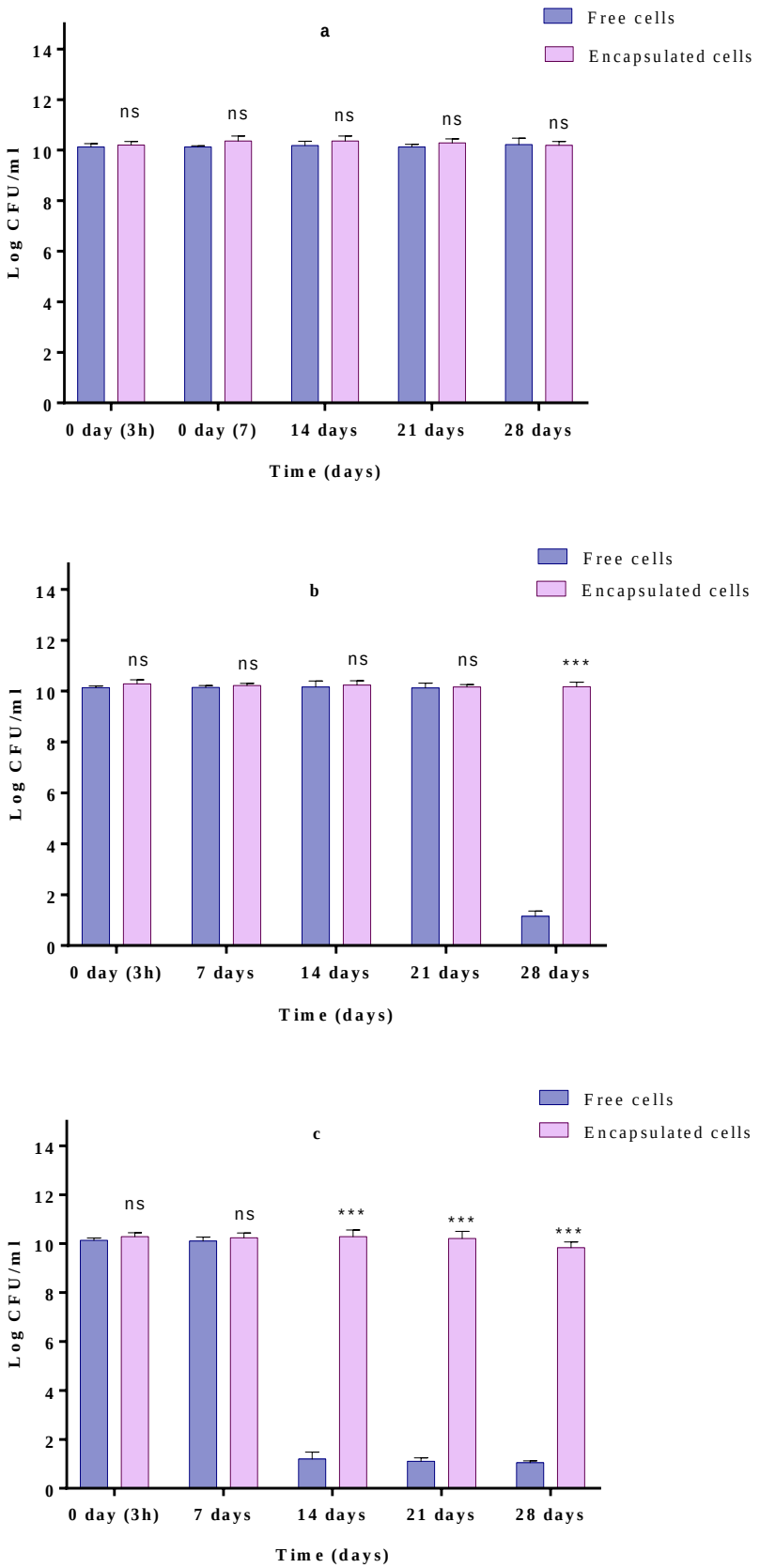


Figure III.9. Viability of free and encapsulated *Lb. plantarum* Q18 at different NaCl concentrations (3%) (a), 6%) (b), 9% (c)) after their storage at 4°C for, 3 h, 07, 14, 21 and 28 days. Results are expressed as Mean ± SD, (n=2). ***p < 0.001, and ns (no significant difference) compared to free cells.

III.3.1.3. Storage in strawberry beverage

The survival of probiotics embedded in the food matrix could be affected during food processing and storage because they are exposed to several stress conditions namely osmotic stress, high temperature, acidic conditions…etc. For example, the presence of high sugar levels in sweetened foods affects their survival due to the high osmotic conditions **(De Prisco *et al.*, 2015)**.

In the present work, the survival of free and microencapsulated cells in strawberry juice was evaluated. A decrease in viability was observed with time for free cells only, however, the number of encapsulated cells remained unchanged, as shown in **figure III.10** for *Lb. plantarum* Q18 and **figure III.11** for *Lb. casei* B1, this means that encapsulation in sodium alginate enhanced the viability of bacteria in sugar stress. The number of *Lb. casei* B1 free cells was reduced by 1.9 Log CFU/ml and 9 Log CFU/ml after 7 and 14 days, respectively, whereas that of encapsulated cells decreased only by 0.2 Log CFU/ml after 14 days with very high significance ($p < 0.001$). For *Lb. plantarum* Q18, viability of free cells decreased after 14 days to reach only 1 Log CFU/ml after 14 days, while for encapsulated cells it was reduced by 0.2 Log CFU/ml after 14 days, this difference was highly significant ($p < 0.001$).

The study of **De Prisco *et al.* (2015)** conducted with apricot jam as a high osmotic pressure food, revealed a significant decline in the survival of free cells compared to microencapsulated ones both subjected to osmotic stress for three hours. The cell number was reduced by about 2 Log cycles for free cells and by about 0.67 Log cycle for microencapsulated ones.

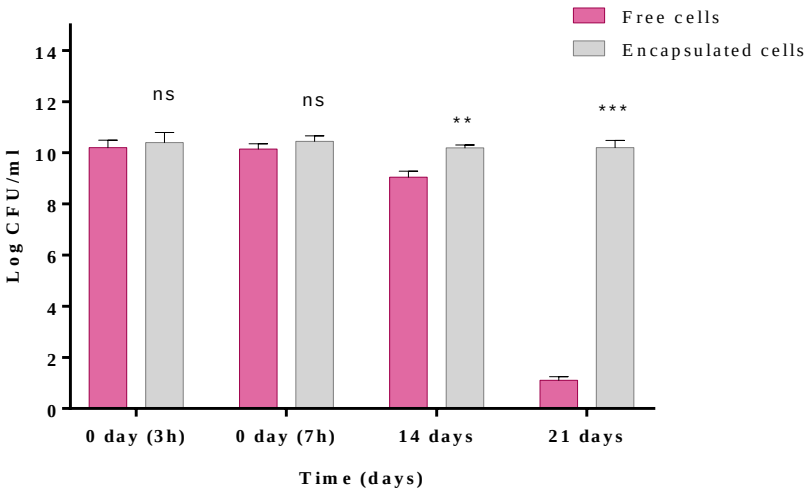


Figure III.10. Viability of free and encapsulated *Lb. plantarum* Q18 after their storage in strawberry juice at 4°C. Results are expressed as Mean ± SD, Sidak's test was used, (n=2).**p < 0.01 , ***p < 0.001, and ns (no significant difference) compared to free cells.

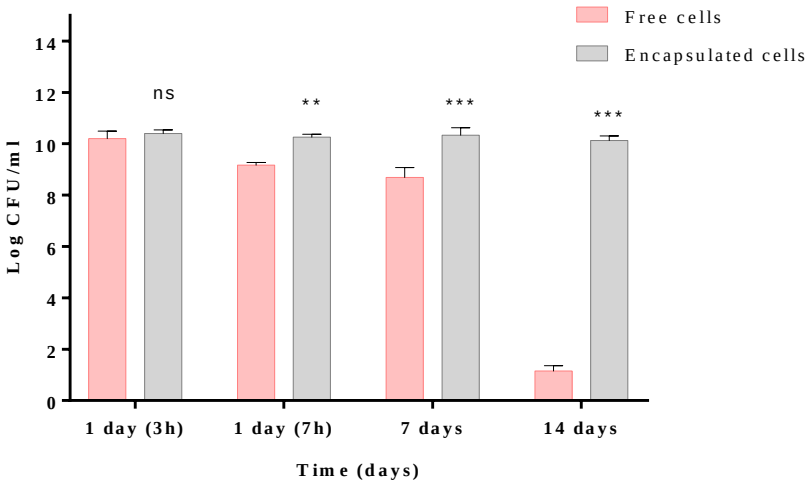


Figure III.11. Viability of free and encapsulated *Lb. casei* B1 after their storage in strawberry juice at 4°C. Results are expressed as Mean±SD, (n=2). Sidak's test was used with significance when **p < 0.01 , ***p < 0.001, and ns (no significant difference) compared to free cells.

III.3.1.4. Viability under simulated gastrointestinal (GI) conditions

This test was performed to evaluate the capacity of the probiotics to overcome the stomachal barrier, for this, free and microencapsulated cells were incubated in simulated gastric juice (SGJ), their respective counts were determined. The initial number of viable free cells (approximately 10.2 Log CFU/ml) decreased to 8 Log CFU/ml and to 8.3 Log CFU/ml for *Lb. casei* B1 and *Lb. plantarum* Q18, respectively following 2h exposure to SGJ **(figures III.12, III.13)**.

These results suggested that free and encapsulated *Lb. plantarum* Q18 and *Lb. casei* B1 cells showed a slight decrease in the number of cells in the acidic environment (pH 2.0). After 4 hours of incubation in simulated intestinal conditions, *Lb. plantarum* Q18 showed the same results for both free and encapsulated cells with a decrease of 2.1 Log CFU/ml. For *Lb. casei* B1, encapsulated cells were reduced in number by also 2.2 Log CFU/ml; however, free cells viability was decrease by 7 Log CFU/ml.

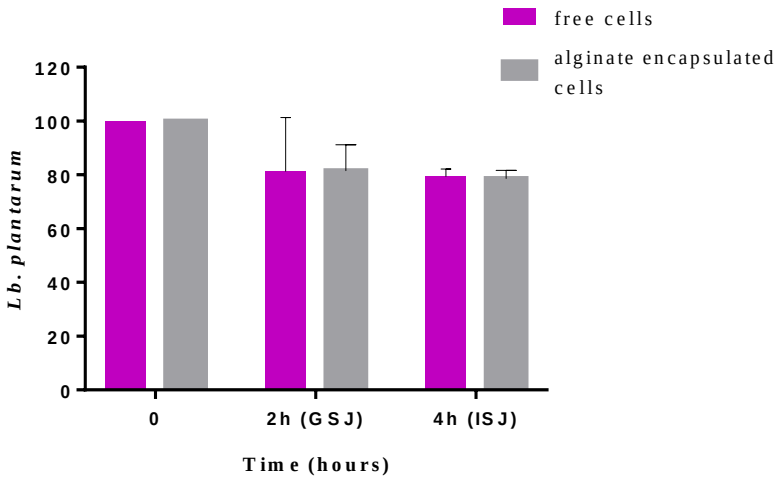


Figure III.12. Viability of *Lb. plantarum* Q18 encapsulated in sodium alginate in gastrointestinal conditions (2h in simulated gastric juice and 4h in intestinal juice). Results were presented as Mean±SD.

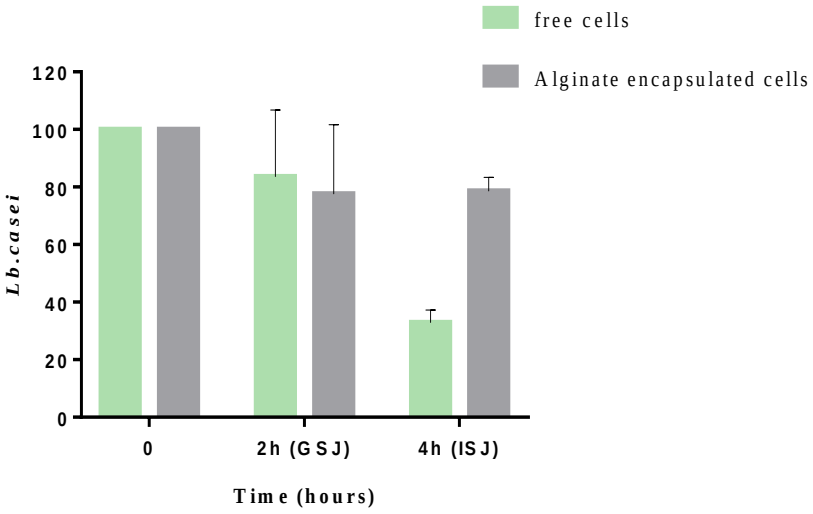


Figure III.13. Viability of *Lb. casei* B1 encapsulated in sodium alginate in simulated gastrointestinal conditions (2h in simulated gastric juice and 4h in intestinal juice). Results were presented as Mean±SD.

III.3.2. Encapsulation of *Lb. plantarum* Q18 with sodium alginate using vibrating technology and chitosan coating

Encapsulation of *Lb. plantarum* Q18 with sodium alginate was also carried out by the extrusion method using the vibrating technology. The obtained capsules were coated with chitosan, and exposed to gastrointestinal conditions. Some drops of freshly obtained encapsulated cells (within normal saline) were deposed on to lame and observed using Nikon optical microscope. **Figure III.14** showed the morphology of alginate microcapsules of *Lb. plantarum* Q18 obtained by vibrating technology under the light microscope. *Lb. plantarum* Q18 clearly appeared surrounded by regular and smooth membranes, they are microcapsules. The perfect spherical shape showed is due to characteristics of the use of vibrating technology where all parameters were controlled from pumping, and hence diameter was 110 ± 5µm.

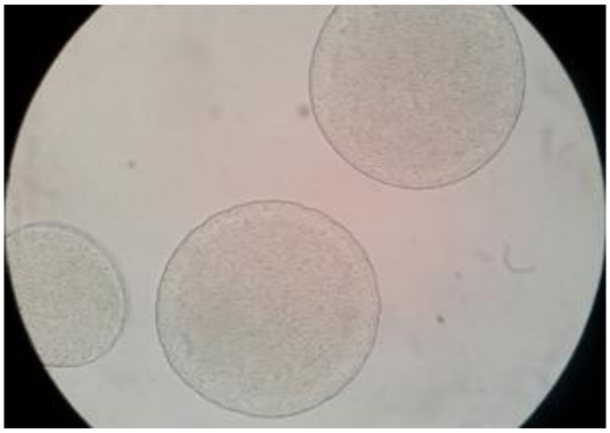


Figure III.14. Optical microscopy images of *Lb. plantarum* Q18 alginate microcapsules at 320 magnification.

The relation between organoleptic characteristics of some foods and microcapsule dimension was reported in literature. Previous studies of **Truelstup-Hansen *et al.* (2002)** mentioned the relation between sensory effect of the product and the beads size, these authors reported that beads with size less than 100 µm are the preferred beads, and bad sensation is related to diameters more than 100 µm.

In the same line, **Audet *et al.* (1988)** reported that the final food product is highly affected by the capsule size; the undesirable parameter affected is the texture when bead size is more than 100µm. The same observation was reported by **Krasaekoopt and Kitsawad (2010)** where microcapsules added to the used fruit juice highly altered its texture. In addition, the

smoothness of yogurt is also reported to be affected when using microcapsules of 300µm **Kailasapathy *et al.* (2006)**.

On the other hand, protective effect against harsh conditions is proportionally related to capsules size, it decreases when bead size decreases and increases when bead size increases too. In fact, negative results of viability in acidic environment were registered with capsules of diameter less than 100µm **(Hansen *et al.*, 2002; Crittenden and Playne, 2008)**.

III.3.2.1. Viability in simulated gastrointestinal conditions

The figure **Figure III.15** showed a decrease in viability for free cells, alginate encapsulated cells and chitosan coated cells in gastric and in intestinal conditions. Chitosan coating beads preserved more viability compared to alginate beads in the both conditions (about 87% and 74% after 2h and 78% and 69% after 4h for chitosan and alginate beads respectively.

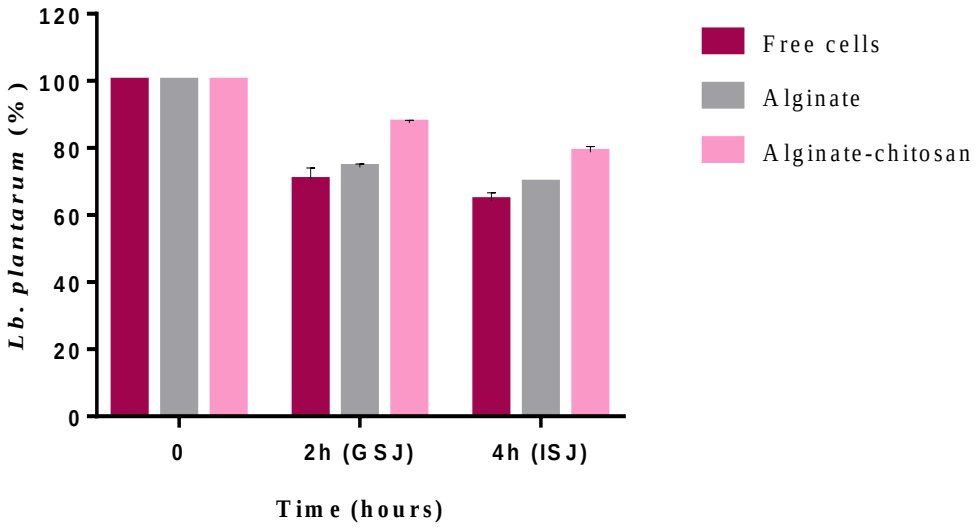


Figure III.15. Viability of *Lb. plantarum* Q18 encapsulated in sodium alginate and coated with chitosan in gastrointestinal conditions (2h in simulated gastric conditions and 4h in intestinal simulated conditions). Results were presented as Mean±SD.

These results can be explained as proposed by **Smidsrod and Skijak-Brack (1990)**, the disintegration of the alginate network in the acidic environment and the chelation of Ca^{+2} by bile, in fact, bile salts act as a chelating agent on Ca^{+2} alginate gel which became easily breakdown and thus cells released **(Smidsrod and Skijak-Brack, 1990)**. However, more protection and so more resistance were observed using another encapsulating matrix such as chitosan and an insoluble complex with bile salts was formed **(Krasaekoopt *et al.*, 2004)**.

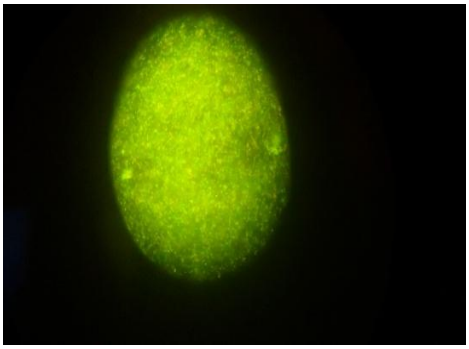
Gbassi *et al.* (2009) showed that chitosan coated alginate capsules were more resistant than alginate ones in simulated gastric fluid.

III.3.2.2. Visualization of microbeads using the fluorescence microscopy

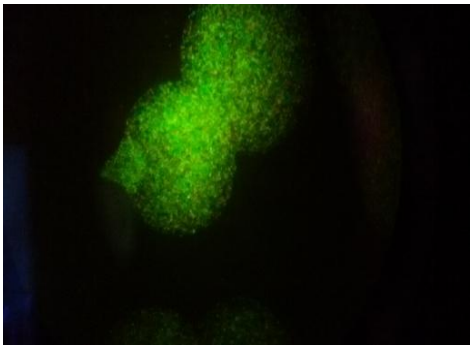
Fluorescence microscope was used to visualize the status of encapsulated and coated capsules after their exposure to gastrointestinal conditions. The principle of this technique is based on the distinction between two colors, green related to healthy (non-damaged) beads and red (for damaged beads). Images were captured by a Nikon Coolpix 4500 Digital Camera equipped with a microscope adapter.

It is well known that harsh conditions such as gastrointestinal environment caused damage to both free and encapsulated cells, hence it is important to show the cell damage by a fluorescence microscopy test at different steps of the microencapsulation process and after the exposure to simulated gastrointestinal conditions as observed in **figure III.16**. Despite of the simulation of the gastrointestinal conditions, results still not really represent the *in vivo* tests because there are many factors affecting survival such as ingested food characteristics, enzymes activity, stress and digestion time **(Hur *et al.*, 2011)**.

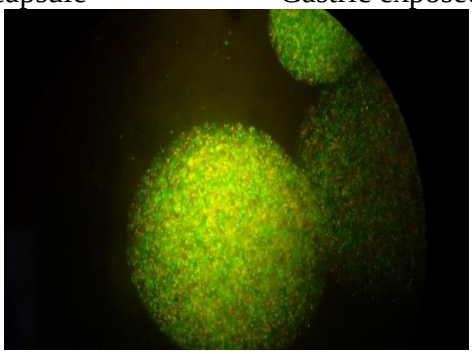
It is clear that after 2h of exposure to gastric conditions, most of capsules in both cases encapsulated in alginate or coated with chitosan appeared green indicating the partially membrane damage, however, the other 4 hours of intestinal fluid exposure cause more damage where both green and red colors appeared mostly in case of alginate.



Alginate capsule

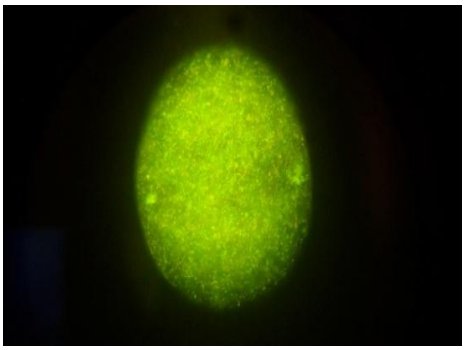


Gastric exposed capsules

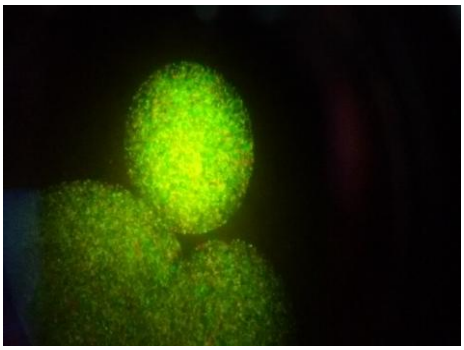


Intestinal exposed capsules

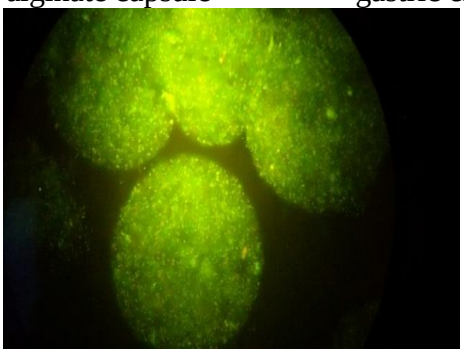
(a)



Chitosan coated alginate capsule



gastric exposed capsules



Intestinal exposed capsules

(**b)**

Figure III.16. Fluorescence microscopy images at 400 magnification of stained *Lb. plantarum* Q18 in alginate (a) and chitosan-alginate microcapsules (b).

However, and as shown in **figure III.16**, capsules with green color in both cases encapsulated with alginate or coated with chitosan in gastric conditions proved the lack of damage of the alginate network or chitosan and this is agree with the findings of **Deprisco *et al.* (2015)**. Moreover, in intestinal environment, alginate capsules appeared partially red which indicates damage due to the presence of bile salts, while chitosan coated beads appeared as green insoluble debris due to the chemical interaction between chitosan and bile salts. Many changes occurred to the beads in acidic conditions, swelling, increase in size, decrease in molecular weight, which lead to an increase in the stability of the beads **(krasaekoopt *et al.*, 2004)**.

III.3.3. Encapsulation of *Lb. casei* B1 and *Lb. plantarum* Q18 with different polymers

III.3.3.1. Bead size and morphology

Extrusion of bacteria in different polymers (matrices) gave beads with the following characteristics where diameters of 20 randomly selected beads of each treatment were measured with a cornier caliper and the number of beads/ml of polymer were presented in **table III.7** and **figure III.17.**

From **table III.7** it is clear that the number of beads varied from one combination to another, in addition, it differed between species. It is of great importance to note that alginate starch mixture gave the highest beads number with both bacteria, followed by chitosan, glycogen, alginate, gum Arabic, carrageenan then locust bean gum with significant difference. The highest beads number was obtained with *Lb. casei* B1 with alginate-starch were we registered 140 ± 4.24 beads and 136±7.07 beads with the same mixture with *Lb. plantarum* Q18 while the lowest one was 68.5±7.78 with locust bean with the same bacterium. Concerning diameters, the largest beads were obtained with carrageenan with more than 2 mm with both bacteria, locust bean also gave beads with considerable diameters and the rest of combinations showed near diameters with no significant difference. The obtained diameters are in the expected diameters interval obtained by the extrusion method by many authors (0.5-3 mm) **(Lotfipour *et al.*, 2012; Pop *et al.*, 2012; Etchepare *et al.*, 2015)**.

It was reported that there was a relation between beads morphology and viability, in fact, oxidative reactions were more effective on rough beads than on smooth ones and gas permeability increases with the presence of any hole, or fracture and decrease with a smooth continuous surface, thus cells are better protected **(Tolun *et al.*, 2016)**, thus we mentioned the shape of our beads. It is important to monitor the size of the beads obtained by encapsulation

knowing that it highly affect the final food product, in fact, beads with small size are not preferred because probiotics within are not well protected. The best interval proposed in order to give the best protection to cells is 100-200 μm **(Nag *et al.*, 2011)**.

Bustamante *et al.* (2017) reported that resistance ability of encapsulated cells against harsh conditions may be bead size dependent. Moreover, bead size also varied according to the combinations of encapsulating matrices and to *Lactobacillus* strain used **(Deandrade *et al.*, 2019),** furthermore, it is also documented that the chemical and physical nature of matrix used to encapsulate bacteria is widely affected their viability **(Etchepare *et al.*, 2016)**.

Table III.7. Number of beads per milliliter of the used polymers and their diameters (mm).

		Carrageenan	Glycogen	Alginate-Chitosan	Alginate	Alginate-Arabic gum	Alginate-Locust bean gum	Alginate-Starch
***Lb. plantarum* Q18**	Number of beads/ml	87.5±3.54 [a]	130±9.90 [c]	129±1.41 [a c]	110.5±7.78 [d]	102.5±6.36 [d]	72.5±7.78 [b]	136±7.07 [c]
	Diameter (mm)	2.015±0.02 [a]	1.115±0.04 [a]	1.11±0.01 [a]	1.37±0.04 [a]	1.535±0.18 [a]	2.2±0.14 [a]	1.165±0.05 [a]
***Lb. casei* B1**	Number of beads/ml	70±4.24 [a]	119.50±3.54	127±1.41 [b]	108.50±2.12	94.00±2.83	68.5±7.78 [a]	140.00±4.24
	Diameter (mm)	2.035±0.11 [a]	1.35±0.35 [a]	1.245±0.21 [a]	1.34±0.03 [a]	1.62±0.14 [a]	1.85±0.07 [a]	1.215±0.16 [a]

Tukey's test was used. Results were considered significant when $p<0.05$. Comparison was carried out between polymers used for encapsulation of each bacterial species. Within each raw, cells labeled with the same letters present no significant difference. Results were presented as Mean±SD, (n=2).

The shape of beads was clearly observed in the **figure III.17**, there was a difference in the shape and in the size, in the smoothness of the beads according to the polymer used.

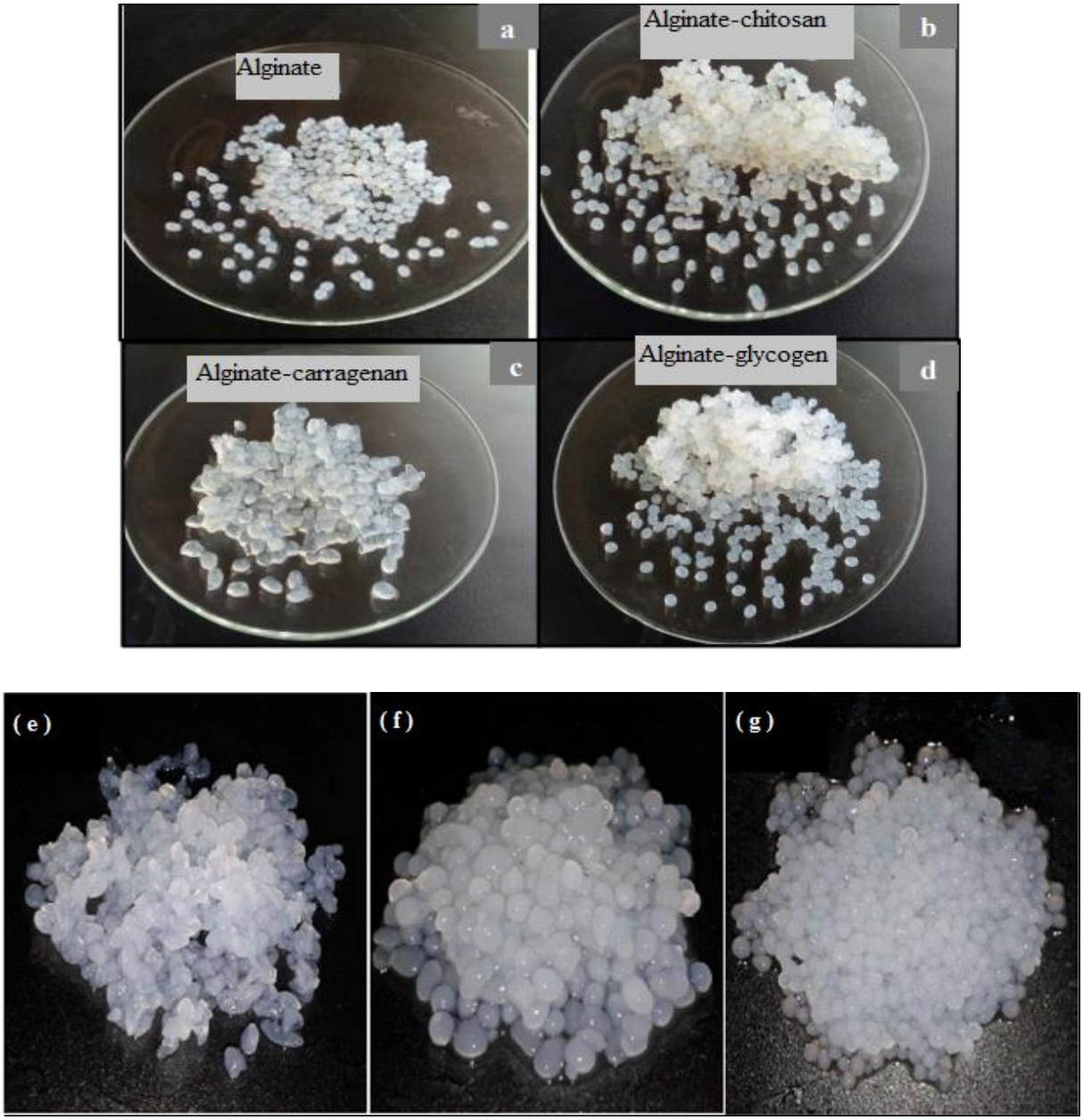


Figure III.17. Macroscopic aspect of the encapsulated *Lb. plantarum* Q18 with the seven polymers; **a**: alginate, **b**: alginate-chitosan, **c**: k-carrageenan, **d**: alginate-glycogen, **e**: alginate-gum Arabic, **f**: alginate-locust bean gum, **g**: alginate-starch.

The shape of the beads carried out after the microencapsulation procedure was generally spheric; sometimes elliptic and cylindric. The alginate beads, alginate-starch and the alginate-glycogen beads had a rounded smooth surface whereas the carrageenan beads had cylindrical and irregular shape and they appear to be the largest beads. The mentioned physical characteristics were explained by many authors; concentration, viscosity, density, elasticity

and the degree of homogeneity of the gel, the distance between the origin of extruding needle and the gelation bath are the reason behind these differences **Wijffels, 2000; Mortazavian *et al.*, 2007)**.

Porous microcapsules may be generated when the cross-linking of alginate by calcium chloride was affected by some factors such as the presence of bacteria and other additives **(Sultana *et al.*, 2000)**. Hence, coating material is used as a second layer to cover these microcapsules and so to fill these pores and thus create a more stable and more impermeable microcapsule **(Shi *et al.*, 2013)**.

III.3.3.2.Viability of free and encapsulated bacteria with polymers

III.3.3.2.1.Viability within 4 weeks of storage at 4°C in normal saline

As shown in **figure III.18**, viability of encapsulated bacteria is better than viability of free cells within 28 days, the best result was registered with gum Arabic (89.27±0.61% to 87.10±0.615%) with 1.19 Log cells reduction and locust bean gum (89.16±0.226% to 86.6±0.76%) with no significant difference, whereas, free cells decreased from (80.28±0.62% to 73.43±0.34%) with 2.72 Log cells reduction. Similarly no significant difference was noted between chitosan, alginate, glycogen and carrageenan; however, the decrease in viability is clear according to time.

Unlike free cells, viability of *Lb. casei* B1 encapsulated in alginate, in carrageenan, in glycerol and coated with chitosan was better than viability of free cells during the fourth weeks **(figure III.19)**. In addition, chitosan coated cells showed the best viability (87.01±0.14%) while free cells viability was (82.91±0.52%). However, viability of cells encapsulated with locust bean, gum Arabic, and starch from the first to the fourth week was lower than viability of free cells. This may due to the inefficiency of encapsulation during the extrusion procedure with these polymers.

In fact, during the extrusion, bacterial cells were entrapped with the used polymer and solidified in a hardening solution and gave beads (capsules) with variable bacterial count. So, depending on the efficiency of encapsulation, the beads contained high or low bacterial cells and also lead to existing of free cells (non encapsulated cells) in calcium chloride, and to destabilization of Ca^{+2} ions distribution around cytoplasm in the phase of capsules hardening **(Reid *et al.*, 2005)**. The decrease in viability during storage in the mentioned polymers was also explained by **(Edgar and Geddes, 1990)**; when interactions occurred between

encapsulated bacteria and their encapsulating polymer this generated metabolites with negative effect on theses bacteria during storage.

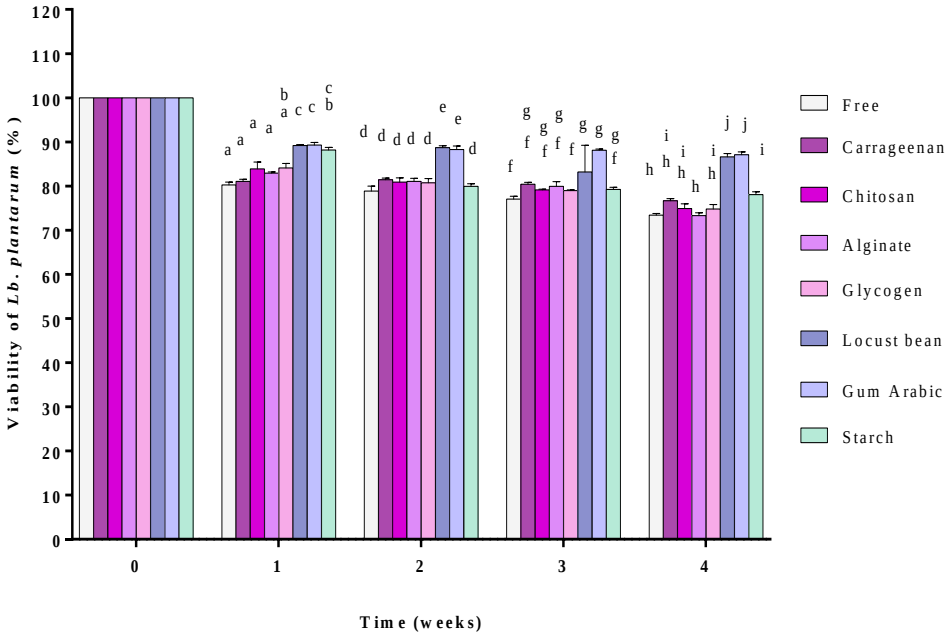


Figure III.18. Viability of free and encapsulated *Lactobacillus plantarum* Q18 in different polymers and incubated in normal saline for four weeks at 4°C. Values shown are Means ±SD (n= 2). Tukey's test was used. Results were considered significant when $p<0.05$. Comparison was carried out between all groups within each week. Bars labeled with the same letters present no significant difference.

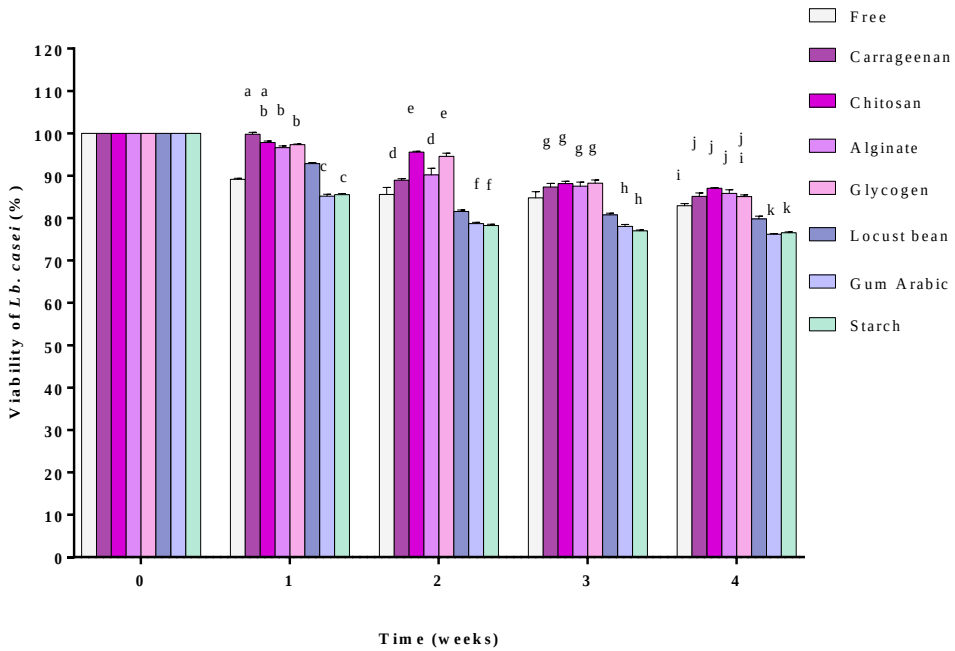


Figure III.19. Viability of free and encapsulated *Lactobacillus casei* B1 with different polymers and incubated in normal saline for four weeks at 4°C. Values shown are Means ±SD (n= 2). Tukey's test was used. Results were considered significant when $p<0.05$. Comparison was carried out between all groups within each week. Bars labeled with the same letters present no significant difference.

III.3.3.2.2. Viability of stored bacteria in pineapple beverage for four weeks

Figure III.20 demonstrated the effect of storage in pineapple juice on the viability of *Lb. plantarum* Q18 encapsulated in different polymers. Storage in pineapple juice gave better results with gum Arabic with about (87.15±0.38)% of viability preserved after 4 weeks, locust bean (86.33±0.38)% and starch (92.37±5)% with no significant difference between them ($P < 0.05$), free cells also remained viable with (73.43±0.34)%. Results of the other polymers were close each to other with no significant difference between them in most cases.

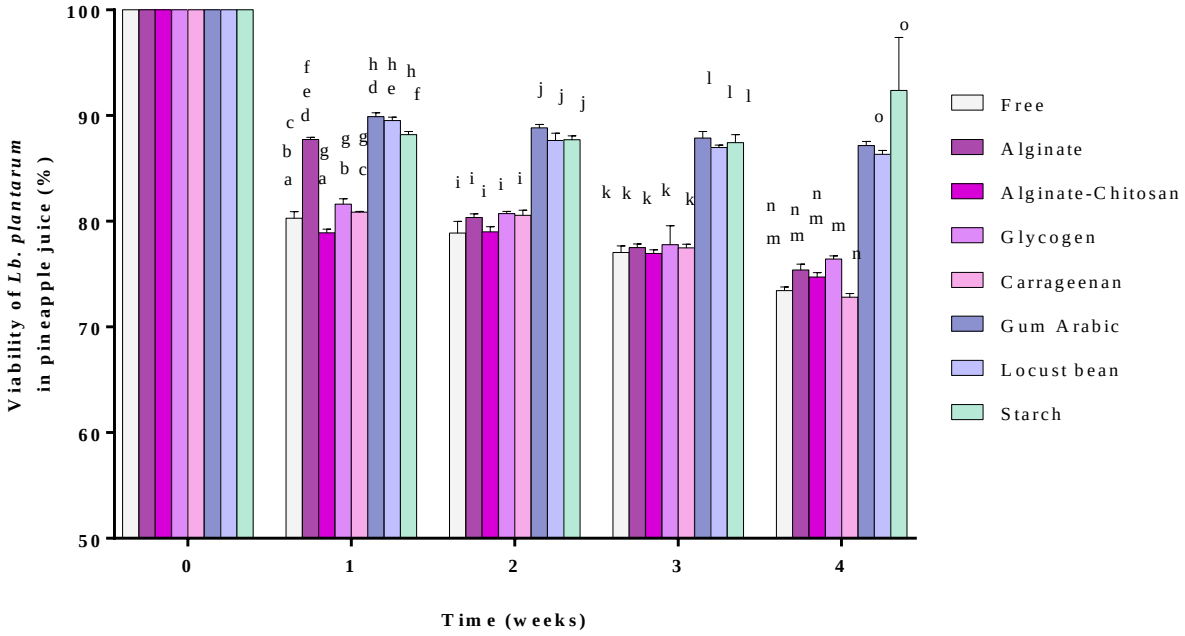


Figure III.20. Viability of *Lactobacillus plantarum* Q18 encapsulated in different polymers in pineapple juice for four weeks at 4°C. Values shown are Means±SD (n=2). Tukey's test was used. Comparison was carried out between all groups within each week. Results were considered significant when $p<0.05$. Bars labeled with the same letters present no significant difference.

As illustrated in **figure III.21**, the previous test was applied on *Lb. casei* B1, the lowest viability was registered with carrageenan in the second, third and in the last week with significant difference (79.28±0.27%) when ($P < 0.05$). During 28 days of storage, alginate, locust bean gum, gum Arabic, starch and glycogen encapsulated cells revealed approximately similar viabilities with no significant difference, encapsulation in sodium alginate supplemented with glycogen showed the best survival with significant difference compared to free cells (82.91±0.52%).

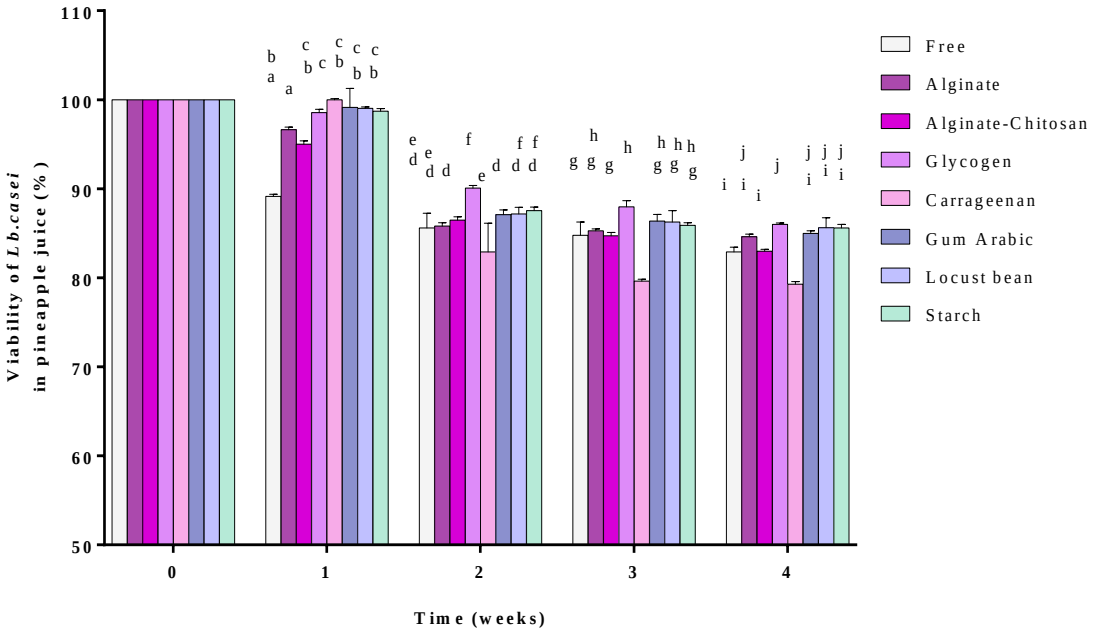


Figure III.21. Viability of *Lactobacillus casei* B1 encapsulated for four weeks in different polymers in pineapple juice at 4°C. Values shown are Means ±SD (n= 2). Tukey's test was used. Results were considered significant when $p<0.05$. Comparison was carried out between all groups within each week. Bars labeled with the same letters present no significant difference.

As summary to our results of storage in both normal saline and pineapple juice, storage appeared species and polymer dependent, for *Lb. plantarum* Q18, locust bean gum, gum Arabic and starch showed the best viabilities in both cases of beverages. For *Lb. casei* B1 and in pineapple juice, also locust bean gum and gum Arabic and starch showed the best viabilities, however, in normale saline, they showed the lowest viabilities. It is also concluded that carrageenan beads showed the lowest load in pineapple stored juice with both isolates. These findings are not only related to the efficiency of encapsulation, or to the juice composition, but also to the chemical structure and interaction between the mixed polymers and bacterial cells and also the porosity of the created beads according to the environmental factors in which they exist **(Klinkenberg *et al,*. 2001; Anal and Singh 2007; Moortazavian *et al.*, 2007)**.

Many authors reported the effect of storage on viability of encapsulated lactic acid bacteria. For example, a mixture of alginate, fenugreek gum and locust bean gum was tested by **Damodharan *et al.* (2017)** to enhance viability of probiotic bacteria in cold storage; viability found to be enhanced, wherein, beads can be stored till 3 months at 4°C. In the same mentioned study, concentrations of (1% alginate, 0.5% locust bean and 0.5% fenugreek gum)

were used. In fact, 2% concentration gave the best results in term of shape, and release reported **Lotfipour *et al.* (2012)**. The same authors documented that no uniform beads shape were obtained when low alginate concentration was used, while the high concentrations lead to a low alginate extrusion through the syringe needle **(Lotfipour *et al.*, 2012)**.

The use of polymers to encapsulate probiotic bacteria and to test their viability during long term storage was reported in different studies, **Khan *et al.* (2013)** reported that viability of encapsulated *B. adolescentis* was preserved for 18 days of storage at 4^{o}C compared to free cells which didn't resist more than week, the same observation was mentioned by **Ortakci and Sert (2012)**, who reported that the period of 4 weeks didn't affect alginate encapsulated *Lb. acidophilus* since only 1 Log cycle was lost. Alginate-chitosan beads viability during 4 weeks storage was better than that of free cells by about one cycle in yogurt and and 1.7 cycles in orange juice **(Krasekoopt *et al*., 2006; Krasaekoopt and Watcharapoka, 2014)** which is explained by the important role of chitosan since it acts as a barrier to the passage of acids and flavonoids into beads **(Vandenberg *et al.*, 2001)**. In a study of **Gandomi *et al.* (2016)**, the coating of alginate beads with chitosan found to enhance viability in both gastrointestinal conditions and for long period of storage, whereas in spite the period of 90 days, free bacteria showed lower viability with 4.5 times lower than that of encapsulated bacteria.

Considering the storage in juices, **Nualkaekool *et al.* (2012)** encapsulated *Lb. plantarum* with alginate then coated them with chitosan, which made these bacteria able to survive up to 42 days at 4°C in pomegranate juice. **Brinques and Ayub (2011)** encapsulated *Lb. plantarum* 11BL in the same mentioned matrices and they reported that these bacteria remained viable up to 38 days. The work of **Simpson (2005)** discussed the effect of the addition of gum Arabic to skim milk and the use of this mixture to spry dried Bifidobacteria, the result indicated that the viability at 4°C during 90 days of storage was higher than 6 Log CFU/g with skim milk, but no enhancement shown with the use of gum Arabic.

Production of juices enriched with encapsulated bacteria was studied by **Pereira *et al.* (2014)** who produced cashew apple juice with encapsulated *Lb. casei* NRRL B-442, viability of the spray dried bacteria using maltodextrin or the mixture of maltodextrin and gum Arabic during storage at 4°C was preserved within 35 days with only 10% loss. Another study of **Dimitrellou *et al.* (2016)** used spray drying method proved the feasibility of encapsulated *Lb. casei* ATCC393 to resist gastrointestinal conditions and to be used to produce fermented milk.

Our results are in agreement with the findings of **Nualkaekul *et al.* (2013)**, the researchers compared the survival of *Lb. plantarum* and *Bifidobacterium longum* in alginate or pectin beads during storage in pomegranate and cranberry juices. They found that the survival of the cells was improved considerably after being entrapped within both matrices. However, free cells of the two strains died after one week of storage in cranberry juice. Furthermore, free cells of *Lb. plantarum* died after 4 weeks and those of *B. longum* after 1 week of storage in pomegranate juice.

During storage, the cell viability decreased for free cells indicated that probiotic cells were highly influenced by the juice composition. In fact, pH, titratable acidity, molecular oxygen, water activity, presence of sugar, salt, artificial flavouring and colouring agent and chemical and microbial preservatives like bacteriocins and hydrogen peroxide are the major factors affecting viability of probiotic in fruit juices said **Chaudary (2019)**. In a similar report, **Vinderola *et al.* (2002)** indicated that the reduced viability in fruit juices may be prinicipally caused by the presence of some inhibitory food additives such as colorings and aoma. In another study, it was shown that the acidic pH of fruit juices ranging from 2.5 and 3.7 with benzoic and lactonic acids may reduce viability of probiotics too **(Sheehan *et al.*, 2007)**.

Moreover, the work of **Shi *et al.* (2013)** reported that no viability loss was detected for *Lb. bulgaricus* coated with milk during the 4^{th} weeks of storage at 4°C while a sharp decrease was noted with free cells to reach only 3.12 Log CFU/ml. Another study that used k-carrageenan as encapsulation material, free and encapsulated *Lb. acidophilus*, were added to tomato juice, encapsulated cells gave better viability of around 6 Log cells/ml after 10 weeks against only 4 Log free cells (diameter was 3 mm) **(Tsen *et al.*, 2008)**. The study of **Gul *et al.* (2019)**, encapsulated *Lb. casei* Shirota in skim milk and gum Arabic using freez-drying technique indicated that encapsulated cells survived well in gastrointestinal conditions and beads were well preserved in temperature of 4^{o}C (only 0.39 Log cycle lost) and 2.43 Log cycles lost at 25^{o}C. Moreover, encapsulated cells survive well and up to 14 days after their addition to dessert.

Results of **Holkem *et al.* (2017)** demonstrated that *Bifidobacterium* BB12 entrapped in alginate didn't success in protecting cells more than 60 days at 25°C. Storage at 4°C, 21°C and at very low temperatures (-20°C, -80°C) was also studied by **Sousa *et al.* (2012)** who followed the extrusion method with alginate for *Lb. acidophilus* Ki, *Lb. casei*-01, and *Lb. paracasei* L26. Viability was well preserved up to 180 days in low temperatures and a decline

in numbers of viable cells during storage was registered at the refrigerated and ambient temperatures. The study of **Polleto *et al.* (2019)** showed that storage at 7°C gave promising results and viability reached 120 days for both beads (alginate and alginate hi maize) with more than 6 Log CFU/g. *Lb. acidophilus* encapsulated also in alginate and resistant starch was able to resist storage of 182 days at 5^{o}C **(Homayouni *et al.*, 2008)**.

III.3.3.2.3. Viability under simulated gastrointestinal conditions

This test was performed to evaluate the capacity of the probiotics to overcome the gastric barrier. Free and microencapsulated cells were incubated in simulated gastric juice (SGJ) and their respective viability was determined at the end of the period of incubation.

The results presented in **figure III.22** showed that the viability of *Lb. plantarum* Q18 in gastric barrier was increased with all polymers except starch compared to the control with no significant difference ($P < 0.05$), and accordingly, the incubation in intestinal conditions showed no difference in term of viability between free cells and encapsulated cells in glycogen, alginate and gum Arabic. The polymers showed the best viability in intestinal conditions were locust bean gum with (100.1±0.65)%, followed by alginate chitosan with viability more than (92.05±0.134%)% with significant difference compared to free cells while viability was (81.37±19.96 and 79.41±2.77) % after 2 and 4h, respectively.

In the case of *Lb. casei* B1, **figure III.23** showed that the viability appeared better in encapsulated cells compared to free cells with no significant difference ($P < 0.05$), while it is significantly enhanced compared to free cells in intestinal conditions, where free cells viability decreased from (83.66±23.10)% in gastric conditions to (32.99±4.22)% in intestinal conditions. Chitosan viability was the highest one in both gastric and intestinal conditions (97.96±0.15%) (about 9.13 Log cells).

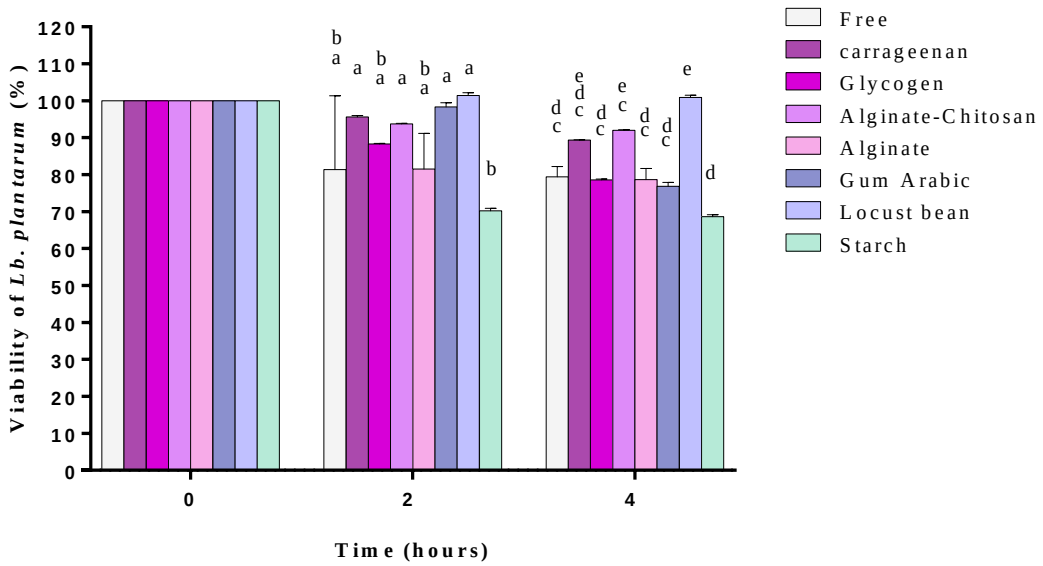


Figure III.22. Survival of encapsulated *Lb. plantarum* Q18 in simulated gastric juice (2h, pH 2.5) and simulated intestinal juice (4h, pH 7.5). Values shown are Means ±SD (n= 2). Sidak's test was used. Comparison was carried out between all groups within each juice. Bars labeled with the same letters present no significant difference; ($P < 0.05$).

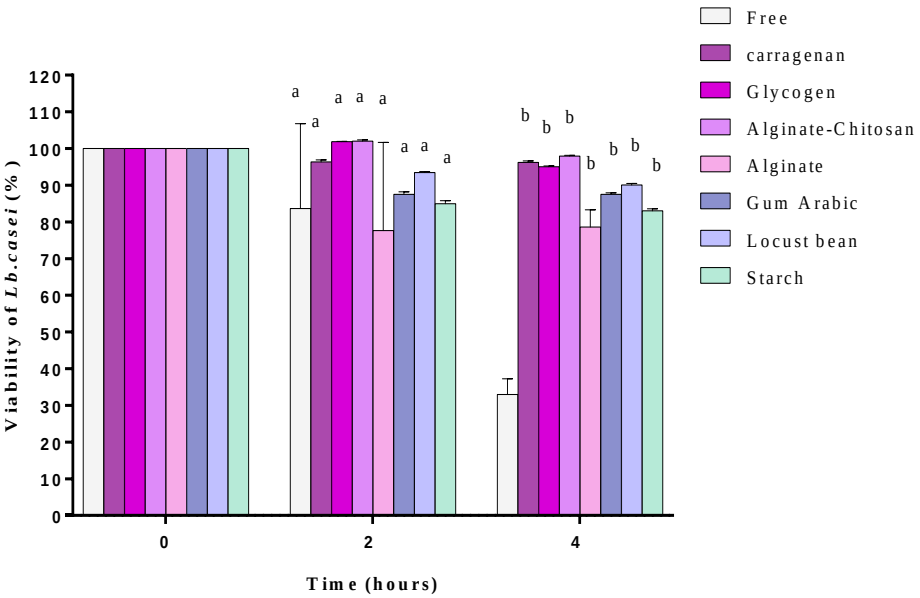


Figure III.23. Survival of encapsulated *Lb. casei* B1 in simulated gastric juice (2h, pH 2.5) and simulated intestinal juice (4h, pH 7.5). Values shown are Means ±SD (n= 2). Sidak's test was used. Comparison was carried out between all groups within each juice. Bars labeled with the same letters present no significant difference; ($P < 0.05$).

Literature published on the encapsulation of lactic acid bacteria with different polymers widely discussed the effect of digestive systems and storage on such cases, agreements were found, contradictions were also reported, for example at the time when some authors noted the enhancement of the viability of the encapsulated bacteria in gastro-simulated conditions, other authors reported negative results. For example, the study of **Corbo *et al.* (2016)** showed that encapsulation of *Lb. plantarum* c19 in alginate succeed in protecting cells in gastrointestinal environment. Other researchers failed to protect probiotic cells through immobilization too; this is the case of **Sultana *et al.* (2000)** and **Gbassi *et al.* (2009),** who reported a lower protective effect of probiotics at pH 2.0. Moreover, **Michida *et al.* (2006)** found that *Lb. plantarum* cells tolerated perfectly the SIJ conditions even at the "free"status.

Negative results were also reported, and viability in digestive system was not found enhanced when encapsulated *Bifidobacteria* in alginate **(Hansen *et al.*, 2002)**. The same observation was reported by **Mokarram *et al.* (2009)** where encapsulated *Lb. acidophilus* and *Lb. rhamnosus* in alginate viabilities were not enhanced **(Etchepare *et al.*, 2016)**. Other authors suggested that the release of bacteria from their encapsulating material may be due to factors related to bacterial cells including biomass distribution inside the bead, cell density as well as biomass distribution near the surface of the beads. Furthermore, interactions between bacterial cells and the polymers are not to be excluded, since they affect the cell release rate **(Klinkenberg *et al.*, 2001; Anal and Singh, 2007)**.

Ding *et al.* (2009) explained the results of encapsulated bacteria in carrageenan and alginate by the similarity of origin of these polymers (seaweed) which gave them similarity in characteristics and protective encapsulating effect. **Sultana *et al.* (2000)** combined starch with alginate in probitic encapsulation and reported that microencapsulation did not improve their survival in bile. It was also reported that the viability of microencapsulated probiotics in bile is highly dependent on the concentration of the encapsulating agent and on the species that is being microencapsulated **(Sohail *et al.*, 2011)**. The publication of **Sabikhi *et al.* (2010)** proved that 2.5h were sufficient for alginate-starch beads to release their probiottic content. High-amylose starch was the most type used for microencapsulation due to its prebiotic properties, in spite its non-specific encapsulant capability. **Chuang *et al.* (2016)** reported that the interactions of calcium ions with the phosphate and hydroxyl groups of starch produce dense structures. The dense structures can be used as an encapsulating material for targeted delivery of probiotics.

The porosity of alginate is the major factor on the failure in the improvement of the survival of probiotic bacteria during acidic condition. Thus, stability of alginate microparticles, was enhanced by its combination with cationic polymers **(Amine *et al.*, 2014)**. The cationic, natural polysaccharide locust bean gum was used to improve the stability of alginate beads **(Cheow *et al.*, 2014)**. It acts as prebiotic to enhance the intestinal microflora and probiotic.

Gum Arabic is complex polysaccharide consist of mixed calcium, magnesium and potassium salt of polysaccharidic acid. It has been reported that gum Arabic has been combined with alginate for delivery of drug **(Nayak *et al.*, 2012)**. Moreover, viscosity of the obtained beads increases when alginate is combined with other polysaccharides and by consequence the synergistic interaction occurs and beads become more stable in low pH solution **(Mohamed *et al.*, 2017)**.

The cross-linking of sodium alginate with calcium ions of the hardening solution is due to its hydrophilic property because it is rich with OH and COOH groups present in its chain **(Chen *et al.*, 2009)**. In acidic environment, the ionic strength was stronger due to the stability of negative and positive charges. However, at pH 7.4, water tends to penetrate into the chain to form hydrogen bond through hydroxylic and carboxylic groups and fills up the space along the chain **(Martingen *et al.*, 1989)**. The same explanation could be given to gum Arabic beads due to the presence of COOH group in its chain **(Nayak and Pal, 2011)**. Moreover, interactions between various functional groups present in the polymers contribute also to the stability of the encapsulating matrices.

In the study of **Dimitrellou *et al.* (2016)** aiming to evaluate the survival of spray-dried microencapsulated *Lb. casei* ATCC 393 cells exposed to simulated gastrointestinal conditions, the researchers reported a higher survival rate of the entrapped cells compared to free ones, both were subjected to simulated gastric juice and bile salts, in this case, a continuous loss in *Lb. casei* viability was observed for free cells after exposure to simulated gastric conditions, the viable cell counts dropped by 4.03 Log CFU/g at pH 2.0. Similar findings were also reported by other researchers working on the same species, indeed, free *Lb. casei* ATCC 393 cells showed a decreased survival rates at low pH values **(Sidira *et al.*, 2010; Li *et al.*, 2011; Xu *et al.*, 2016)**. In addition, spray-drying microencapsulation of *Lb. plantarum* provided an efficient protective effect facing bile salts solutions when compared to freeze-drying, as highlighted by **Rajam *et al.* (2012)**. Moreover, **Mandal *et al.* (2006)** reported also an improved viability of *Lb. casei* NCDC-298 cells after being exposed to 1% and 2% bile salts

for 12 h, this improvement is resulting from alginate encapsulation, and it was proportional to the polymer concentration. In the same context, the study of **Brinques and Ayub (2011)**, using *Lb. plantarum* BL01, reported that incubation in simulated gastric medium showed no change in cell viability compared to free cells, in other words, viability of both free and immobilized cells was deeply affected by the incubation conditions, excluding the protective effect of the polymeric matrix.

Efficiency of encapsulation is related to many factors, mentioning the concentration of the used polymer **(Sandoval-Castilla *et al.*, 2010)**, the nature of the used polymer, and the procedure followed **(Weijmer *et al.*, 2002)**. The study of **Forautan *et al.* (2017)** provided optimizing results about enhancement of viability of *Lb. casei* PM01 in alginate encapsulated beads, in agreement with what is published by **Mandal *et al.* (2006)** about the increase of viability of *Lb. casei* NCDC-298, when encapsulated in sodium alginate. In spite the well-known characteristics of alginate, the principal drawback appeared when simulating gastrointestinal conditions because of its instability in phosphate environment and thus, liberation of bacteria from beads increased, to overcome this obstacle, the blend of sodium alginate with other polysaccharides and encapsulating material or covering the alginate beads with coating materials seems to be of great interest (**Forautan *et al.* 2017)**.

The chitosan polymer can protect cells by the interaction between negatively charged particles of alginate and positively charged particles of chitosan, rendering beads more stable and more resistant to deleterious environmental factors. This can explain our results, and it is agreed with findings of the study of **Chavari *et al.* (2010)**, which reported that the viability of chitosan encapsulated *Lb. gasseri*, and *Bifidobacterium bifidum* were increased in digestive like conditions. Furthermore, *Lb. casei* coated with chitosan exhibited more viability than the uncoated (only alginate encapsulated) cells **(Yu *et al.*, 2001)**. Many authors mentioned that chitosan not only enhanced the viability of alginate encapsulated bacteria but also participate into encapsulated alginate beads themselves. In fact, in the intestinal environment, where bile salts exist, chitosan react with them and reduce their permeability inside the beads ensuring so, a double protection as a second thick layer **(Murata *et al.*, 1999)**.

Concerning, release of encapsulated probiotic bacteria in intestinal simulated fluid, it was discussed by several authors using several bacterial species and several polymers. In the study of **Shi *et al.* (2013)**, capsules of carrageenan-locust bean beads release their bacteria fastly in intestinal juice, while detected viability was 8 Log CFU/g. In the same way, *B. adolescentis*

encapsulated in alginate and coated with a pea protein release their content slowly, noting that the presence of $NaHCO_3$ serves as a destabilizing agent for alginate **(Klemmer *et al.*, 2011)**.

The use of the bacterium *Lb. acidophilus* and alginate as encapsulation matrix, and rice bean, inulin and resistant starch (hi-maize) as additional matrices by external gelation led to formation of beads with different diameters ranging from about 80µm with alginate to 117.7µm with rice bran, viability appeared to be enhanced in gastrointestinal conditions **(Poletto *et al.*, 2019)**. Likewise, in the work of **Gebara *et al.* (2013)** encapsulated *Lb. acidophilus* survived better than free cells which lost 6 Log cycles.

Also, **Shi *et al.* (2013)** used extrusion method to encapsulate *Lb. bulgaricus* with milk then coated beads with carrageen-locust bean gum mixture and found that encapsulation enhanced viability compared to free cells in gastrointestinal conditions. This result is in agreement with findings of **Etchepare *et al.* (2016)** where hi-maize encapsulated beads obtained by extrusion remained viable through 6h incubation in gastrointestinal conditions. Furthermore, the publication of **Nunes *et al.* (2018)** showed that free *Lb. acidophilus* cells were less protected than encapsulated cells with hi maize, inulin and trehalose.

Tow lactobacilli were used in the study of **De andreade *et al.* (2019)**, *Lb. plantarum* and *Lb. brevis*, the encapsulating materials were whey proteins, whey proteins and inulin or maltodextrins, the conclusion was that *Lb. plantarum* was more viable than *Lb. brevis* in gastrointestinal conditions and that *Lb. brevis* count was less than 6 Log CFU/g. Electro spraying procedure was used to encapsulation *Lb. plantarum*, with alginate and citric pectin were used, again, encapsulation proves its ability to provide beads a considerable resistance against gastrointestinal conditions with reduction loss of 3 Log CFU/g **(Cogetto *et al.*, 2016)**.

Conclusion

Conclusion

Nowadays, microencapsulation through different techniques and within different polymers gets a lot of attention as a promising technology for lactic acid bacteria protection. The emphasis itself comes firstly from the advantages of these later, including production of nutrients and co-factors, competition with pathogens and stimulating the host immune response, and secondly from its ability to cover these living cells, giving them more chances to exert their health benefits, since they remain far from the deleterious environmental factors during both processing and digestion, thus remaining viable till reaching colon where they act. In this context, we underlined our aims throughout this study by isolation of lactic acid bacteria from the Algerian traditional cheese "Klila", then selection of bacteria with the best probiotic and technological properties. The selected bacteria were encapsulated in sodium alginate mixed to different polymers and viability under storage at 4°C and under gastrointestinal conditions was tested. Our results indicated that "Klila" contains different species of lactic acid bacteria, as confirmed by genetic identification through 16S rRNA sequencing, namely *Lb. plantarum* as a dominant species, *Lb. pentosus*, *Lb. casei* and *Lb. brevis*. Few isolates are considered to have some technological traits principally acidifying activity and proteolytic activity. No isolate exhibited lipolysis or exopolysaccharide production. Antagonism was tested against *E. coli* ATCC 25422, *S. aureus*, *B. subtilis*, *L. monocytogenes*, and *Salmonella* sp. All our bacteria showed antagonist activity against at least one pathogen, while two of the isolates displayed inhibition activity against all tested organisms (*Lb. plantarum* Q18 and *Lb. casei* B1). In addition, resistance was tested against two antibiotic groups: cell wall inhibitors (Penicillin G, Amoxicillin) and protein synthesis inhibitors (Streptomycin, Gentamycin), and Colistin sulfate. Most of our isolates were sensitive to the five antibiotics used with almost equal diameters, where the largest one (27.75 ± 0.35 mm) was obtained with Q28 against Gentamycin and the smallest one (6 mm) with Q8 against Colistin sulfate. Results also showed that *Lb. casei* B1 and *Lb. plantarum* Q18 as selected strains exhibited good auto-aggregation and adhesion properties in addition to their tolerance to gastrointestinal simulated conditions and bile salts.

Moreover, our results showed that, in most of cases, encapsulated cells exhibited better viabilities compared to non-encapsulated (free) ones with significant differences ($p<0.05$). *Lb. plantarum* Q18 encapsulated in locust bean or in gum Arabic gave the highest viabilities in pineapple beverage with highly significant differences compared to free cells stored in the same conditions. However, *Lb. casei* B1viability was the best within chitosan followed by alginate, with significant differences compared to free cells. Moreover, viability in pineapple juice was similar in locust bean, gum Arabic, glycogen and alginate stored at 4°C. Storage in strawberry beverage showed

that encapsulated cells survive better than free cells.

In gastrointestinal conditions, viability was again better with locust bean gum followed by chitosan with *Lb. plantarum* Q18 while it was better with chitosan, glycogen and carrageenan in the case of *Lb. casei* B1. Viability of encapsulated *Lb. plantarum* B1 cells in alginate was also enhanced at high salt concentrations.

As perspectives

Throughout this work, our objective in isolation of lactic acid bacteria from "Klila" was achieved. The selected bacteria with good probiotic and technological traits were encapsulated, stored and exposed to gastrointestinal conditions. However, this work remains an initiative for many other purposes, such as:

- Using other techniques of encapsulation like spray drying, emulsion, coacervation with the same studied strains and polymers and also with other polymers.
- Using other non-polysaccharide polymers (proteins for example).
- Monitoring each step from encapsulation to the last day of storage by scanning electron microscopy
- Applying these encapsulated cells in various food matrices, comparing their viabilities and selecting the best proposed functional food.
- Optimizing factors affecting microencapsulation (such as concentration of polymers, the best ratio in case of mixture, $CaCl_2$ molarity, trying other hardening agents).
- *In vivo* application in animal models.

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Appendix

Appendixes

Table1. Physiological characteristics of isolates

Isolates	Growth at differenT°		ADH	Type of Fermentation	Growth at NaCl	
	10°C	45°C			4%	6.5%
K1	-	+	-	Homo	+	-
K2	-	+	-	Homo	+	-
K3	-	+	-	Homo	+	-
K4	-	+	-	Homo	+	+
K5	-	+	-	Homo	+	+
K6	-	+	-	Homo	+	+
K7	-	+	+	Hetero	+	+
Q18	-	+	-	Homo	+	+
K9	-	+	-	Homo	+	+
K10	-	+	-	Homo	+	+
K11	-	+	-	Homo	+	+
K12	-	+	-	Homo	+	+
K13	-	+	-	Homo	+	+
Q14	-	+	-	Homo	+	+
B1	-	+		Homo	+	+
Q5	-	+	-	Homo	+	+
K17	-	+	-	Homo	+	-
K18	-	+		Homo	+	-
KBM2	-	+	+	Hetero	+	+
Q28	-	+	-	Homo	+	+
Q8	-	+	-	Homo	+	+
Q1	-	+	-	Homo	+	+
Q3	-	+	-	Homo	+	-
Q9	-	+	-	Homo	+	+
Q20	-	+	-	Homo	+	-
Q30	-	+	-	Homo	+	-
Q2	-	+	-	Homo	+	-
Q6	-	+	-	Homo	+	-
K29	-	+	-	Homo	+	-

Table.2. Carbohydrates fermentation

Biochemical identification	Molecular identification	Isolates	Xylose	Glucose	Levulose	Mannose	Sorbose	Cellubiose	Threalose	Inositol	Sucrose	Galactose	Raffinose
Lb.acidophilus		K1	_	_	+	+	±	±	+	+	+	+	±
Lb. plantarum		K2	±	±	±	+	+	+	+	+	+	+	+
Lb.helviticus		K3	_	_	+	+	+	_	+	+	_	+	_
Lb.helviticus		K4	_	_	+	+	+	_	+	+	_	+	_
Lb.plantarum		K5	±	+	±	+	+	+	+	+	+	+	+
Lb.plantarum		K6	±	+	±	+	+	+	+	+	+	+	+
Lb.brevis		K7	+	+	+	_	+	_	_	+	_	±	+
Lb.plantarum	*Lb. plantarum*	Q18	±	+	±	+	+	+	+	±	+	+	+
Lb.plantarum		K9	±	+	±	+	+	+	+	+	+	+	+
Lb.plantarum		K10	±	+	±	+	+	+	+	+	+	+	+
Lb.plantarum		K11	±	+	±	+	+	+	+	+	+	+	+
Lb.plantarum		K12	±	+	±	+	+	+	+	+	+	+	+
Lb.plantarum		K13	±	+	±	+	+	+	+	+	+	+	+
Lb.acidophilus	*Lb.plantarum*	Q14	±	±	±	+	+	+	+	+	+	+	+
Lb.casei	*Lb. casei*	B1	_	+	+	+	±	+	+	±	+	+	_
Lb.plantarum	*Lb. pentosus*	Q5	+	+	±	+	+	+	+	+	+	+	+
Lb.plantarum		K17	±	+	±	+	+	+	+	+	+	+	+
Lb.casei		K18	_	_	+	+	±	+	+	±	+	+	_
Lb.fermentum	*Lb. brevis*	KBM2	±	+	+	_	+	±	_	+	±	+	+
Lb.plantarum	*Lb. plantarum*	Q28	+	+	±	+	+	+	+	+	+	+	+
Lb.plantarum	*Lb. plantarum*	Q8	±	±	±	+	+	+	+	+	+	+	+
Lb.acidophilus	*Lb. plantarum*	Q1	±	_	±	+	+	±	+	+	+	+	+
Lb.plantarum	*Lb. plantarum*	Q3	+	+	±	+	±	±	+	+	+	+	+
Lb.acidophilus	*Lb. plantarum*	Q9	_	_	±	+	+	+	+	+	+	+	+
Lb.acidophilus	*Lb. plantarum*	Q20	_	_	±	+	+	+	+	+	+	+	+
Lb.plantarum	*Lb. plantarum*	Q30	+	+	+	+	+	+	+	+	+	+	+
Lb.plantarum	*Lb. plantarum*	Q2	+	±	+	+	+	+	+	+	+	+	+
Lb.plantarum	*Lb. plantarum*	Q6	±	±	+	±	+	+	+	+	+	+	+
Lb.plantarum		K29	+	+	+	-	+	+	+	+	+	+	+