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Encapsulation of Nisin producing strain of *Lactococcus lactis* for active packaging development

Mariam Bekhit

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Université de Lorraine
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Ecole Doctorale Sciences et Ingénierie des Ressources, Procédés, Produits, Environnement
(RP2E)
Laboratoire d'Ingénierie des Biomolécules (LIBio)

THESE

Présenté devant L'Université de Lorraine
Pour obtenir le grade de Docteur de l'Université de Lorraine
Spécialité : Procédés Biotechnologiques et Alimentaires

Par

Mariam BEKHIT

**Encapsulation de la souche *Lactococcus lactis*, productrice de nisine,
pour le développement d'emballages actifs.**

**Encapsulation of Nisin producing strain of *Lactococcus lactis*
for active packaging development**

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2- **Bekhit, M.**, Sánchez-González, L., Ben Messaoud,G., and Desobry. S; 2015, Physical properties and antilisterial activity of bioactive films containing alginate/pectin composite microbeads with *Lactococcus lactis subsp lactis*.

International Oral Presentations:

1- Bekhit, M., Sánchez-González, L., Ben Messaoud,G., and Desobry. S; 2015, Encapsulation of *Lactococcus lactis subsp. lactis* ATCC 11454 in microcapsules based on biopolymers”
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Posters :

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2- Bekhit, M., Sánchez-González, L., Ben Messaoud,G., and Desobry. S; 2014 “Encapsulation of *Lactococcus lactis subsp. lactis* ATCC 11454 in microcapsules based on biopolymers Alginate/Xanthan” Seminaire annuel 2014 école doctorale RP2E, Nancy, France.

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List of Abbreviations

AFM	Atomic force microscopy
ATR-FTIR	Attenuated total reflectance Fourier transform infrared
A/P	Alginate/pectin
CAP	Cellulose acetate phtalate
CFU	Colony forming unit
CLSM	Confocal Laser Scanning Microscopy
DE	Degree of esterification
DSC	Differential scanning calorimetry
E	Elongation
EM	Elastic modulus
FBRM	Focused beam reflectance measurement
FFD	Film forming aqueous dispersions
HLC	Human like collagen
HMP	High methoxyl pectin
HPMC	Hydroxypropylmethyl cellulose
LAB	Lactic Acid Bacteria
<i>L. lactis</i>	<i>Lactococcus lactis</i> subsp. <i>lactis</i>
<i>L. monocytogenes</i>	<i>Listeria Monocytogenes</i>
LMP	Low methoxyl pectin
<i>M. flavus</i>	<i>Micrococcus Flavus</i>
NaCMC	Sodium carboxymethyl cellulose
OP	oxygen permeability
rpm	Revolutions per minute
SEM	Scanning Electron Microscopy
SPOS	Single particle optical sensing
SLS	Static light scattering
TEM	Transmission Electron Microscopy

TS	Tensile strength
TSB	Tryptone Soy Broth
wt	Weight
WVP	Water vapour permeability
w/w	Weight/weight

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I. Introduction

Introduction

La préoccupation croissante des consommateurs pour la santé devient un facteur essentiel de l'innovation dans de nombreux domaines de l'industrie alimentaire. Une des problématiques les plus importantes est le remplacement des ingrédients issus de synthèse chimique par des ingrédients d'origine naturelle. Les conservateurs naturels sont ainsi préférés aux autres et l'utilisation de métabolites antimicrobiens naturellement produits par les bactéries lactiques (LAB), comme principalement la nisine, permettent une réduction des pathogènes bactériens d'origine alimentaire telles que *L. monocytogenes*, *S. aureus*, *Clostridium botulinum*, *Clostridium perfringens*, *Bacillus cereus*, et d'autres. La nisine est un peptide antimicrobien contenant des acides aminés et des résidus insaturés lanthionine qui peuvent inhiber la croissance d'une large gamme de bactéries gram-positives et qui, par conséquent, est utilisé comme agent de conservation dans différents produits alimentaires.

Pour éviter d'incorporer les LAB directement dans les aliments ou elles se développeraient très rapidement, une voie consiste à les placer dans le matériau d'emballage et de permettre la migration de la nisine active vers l'aliment selon le concept de l'emballage actif. Cela est envisageable par le biais de LAB piégés dans des microbilles et incorporés dans des films de biopolymères. Les films de polymères bioactifs ainsi aurait également pour effet de ralentir la croissance bactérienne tout en permettant la synthèse de nisine pendant un stockage prolongé. Cette piste de recherche est à l'origine du travail de thèse développé ici. Le groupe des LAB contient des espèces typiques appartenant aux genres *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus*, *Oenococcus*, *Enterococcus*, et *Leuconostoc*. Ces LAB sont considérés comme des bactéries positives jouant un rôle dans le domaine de la santé tel que la protection de l'intestin par sa résistance aux bactéries pathogènes. En particulier, *Lactococcus lactis* subsp. *lactis* (*L. lactis*) a montré une activité antagoniste vis-

à-vis de *Listeria spp* qui est un contaminant majeur des aliments réfrigérés. *L. lactis* peut produire de la nisine.

L'inclusion de cette bactérie dans des microbilles afin de les protéger d'un environnement hostile et contrôler la libération de nisine commence à être étudiée au niveau international. Il existe différents polymères utilisés dans l'encapsulation (principalement des polysaccharides et protéines) et de nombreuses techniques d'encapsulation. La sélection d'une technique compatible avec la stabilisation et le maintien en vie de bactéries lactiques est assez complexe. La méthode la plus utilisée pour la production de microcapsules contenant des probiotiques est l'extrusion, en raison de la simplicité de son fonctionnement, son faible coût et les conditions de formation appropriées vis-à-vis de la viabilité des bactéries.

LITERATURE REVIEW

Chapter I: Litterature Review

1. Introduction

The encapsulation process aims to entrap a specific component within a matrix (proteins, polysaccharides, etc.). There are examples for component that need to be encapsulated for use in the food industry, such as flavors to control aroma release, antimicrobials to protect from spoilage, antioxidants to increase nutritional value and delay chemical degradation, vitamins to increase their bioavailability, or even probiotics (as lactic acid bacteria) to improve food value

1.1. Capsules roles and encapsulation objectives

Encapsulated probiotics is used to protect the cells against a harsh environment, control their release, avoid alteration in stomach and improve LAB viability in products (Burgain, Gaiani, Linder, & Scher, 2011) ; (Nedovic, Kalusevic, Manojlovic, Levic, & Bugarski, 2011). Therefore, particles with diameters of a few μm to a few mm are produced. There are various names for the substance used for encapsulation, as coating, membrane, shell, carrier material, wall material, external phase or matrix. The matrix used for prepare microcapsules in food processes should be food grade and able to protect the LAB entrapping in matrix (Nedovic et al., 2011).

1.1.1. The different structures of capsules

Microencapsulation technique occurs typically in three phases. The 1st phase aims the integration of bioactive component in a matrix, which can be liquid or solid. In the case of liquid core, integration is a dissolution or a dispersion in the matrix. If the core is solid, the incorporation is an agglomeration or an adsorption. The 2nd phase distributes liquids while powder dissolved in a matrix. The last step, consists in stabilizing the capsules by chemical (polymerization), physicochemical (gelation) or physical (evaporation, solidification, coalescence) process (Passos & Ribeiro, 2009).

Microcapsules can be ranked in 4 categories (Fig.1):

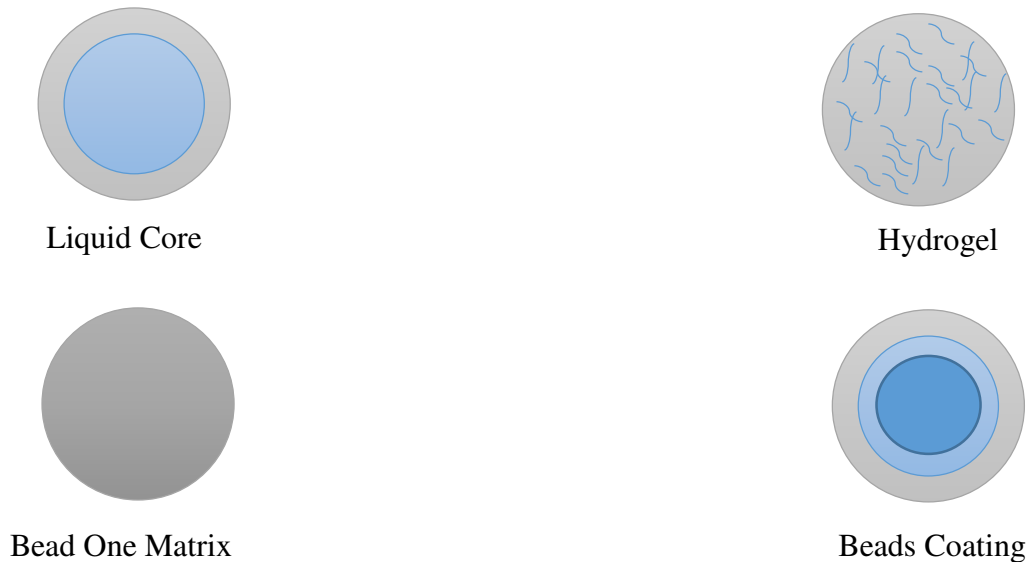


Figure 1 : The different types of capsules

- (i) matrix-core/shell microcapsules processed by getting biopolymers drops in a solution containing a bivalent ion followed by treatment of the surface with a polycation “multi-step technique” (Murua, de Castro, Orive, Hernández, & Pedraz, 2007); (Willaert & others, 2006) .
- (ii) Liquid-core/shell microcapsules processed by dropping a cell suspension containing bivalent ions into a biopolymers solution “one-step technique”.
- (iii) Cells-core/shell microcapsules (coating).
- (iv) Hydrogel microcapsules in which cells are hydrogel-embedded.

Many attempts has been over the years to improve the microencapsulation techniques such as correct biomaterial characterization and purification, improvements in microcapsules production procedures (Wilson, Cui, & Chaikof, 2008).

1.2. Application to cell protection and release

Capsules prevents the cell release and increases mechanical and chemical stability (Overgaard, Scharer, Moo-Young, & Bols, 1991). The capsules are often obtained by coating technique (negative charge polymers as “alginate” coated by positively charged polymers as “chitosan”) that enhances stability of the gel (Smidsrød, Skja, & others, 1990) and provides a barrier to cell

release (Dumitriu, 1996), (Gugliuzza, 2014), (Zhou, Martins, Groboillot, Champagne, & Neufeld, 1998). (Tanaka, Irie, & Ochi, 1989) reported the coating of gel capsules by a cell-free alginate gel layer.

Cross-linking with cationic polymers to improve the stability of microcapsules (Kanekanian, 2013). (Kolot, 1988); (Garti & McClements, 2012); (Kwak, 2014) develops a membrane around the beads to minimize cell release, and produces stronger microcapsules. The reaction of the biofunctional reagent with chitosan membrane results in bridge formation linking the chitosan molecules. The length of the bridge depends on the type of cross-linking agent (Hyndman, Groboillot, Poncelet, Champagne, & Neufeld, 1993).

For dry capsules, incorporation of cryoprotectants such as glycerol enhances survival of encapsulated cells after lyophilisation and rehydration (Lakkis, 2008). (Aziz Homayouni, Azizi, Javadi, Mahdipour, & Ejtahed, 2012) presented that the survival of bifidobacteria increased significantly. (Sheu, Marshall, & Heymann, 1993) reported *Lb. delbrueckii ssp. bulgaricus* survival up to 90% because these agents reduced ice crystal formation by water binding. The capsules with glycerol also exhibited a 43% decrease capsules size, due to higher alginate concentration per unit volume in the capsules with glycerol binding with water.

2. Polymers used for encapsulation

The selection of a biopolymer or combination of biopolymers depends on many factors: the desired physicochemical and functional properties of the particles (e.g., size, charge, polarity, loading capacity, permeability, degradability, and release profile), the properties of the biopolymers (e.g., charge, polarity, and solubility), and the nature of any enclosed active ingredient (e.g. charge, polarity, solubility, and stability) (Joye & McClements, 2014). Several matrix are used to offer a large range of properties adapted to the entrapped bacteria.

2.1. Carbohydrates

2.1.1. Alginate

Alginate hydrogels are extensively used in microcapsules with probiotics because of their simplicity, non-toxicity, biocompatibility and low cost (Rowley, Madlambayan, & Mooney, 1999) (Krasaekoopt, Bhandari, & Deeth, 2003). Alginate is a linear heteropolysaccharide extracted from different types of algae with two structural units consisting of D-mannuronic (M) and L-guluronic acids (G) (Fig. 2). Depending on the source, the composition and the sequence in D-mannuronic and L-guluronic acids vary widely and influence functional properties of the material. G-units have a bucked shape while M-units tends to extended band. Two G-units aligned side-by-side result in the formation of a hole with specific dimension, which is able to bind selectively divalent cations.

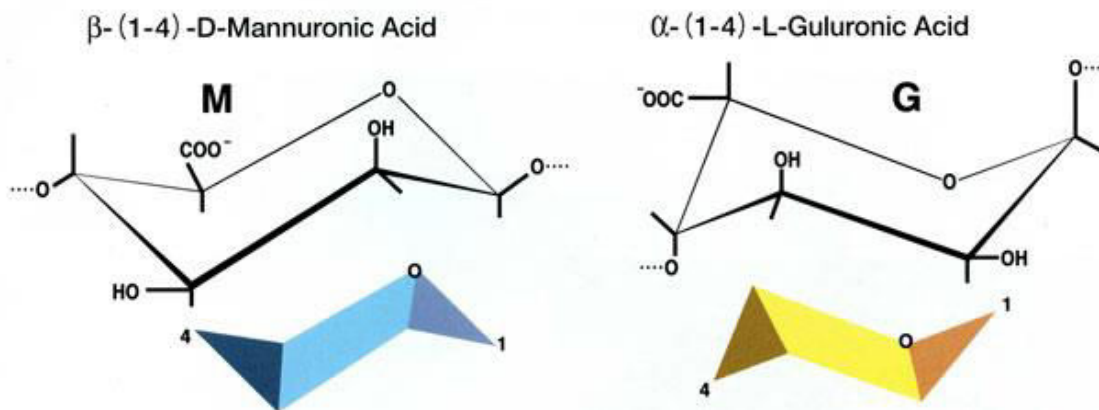


Figure 2: Alginate structure.

To prepare alginate capsules, sodium alginate droplets fall into a solution containing a multivalent cations (usually Ca^{+2} in the form of CaCl_2). The droplets form gel spheres instantaneously, entrapping the cells in a three dimensional structure due to a polymer cross-linking by exchange of sodium ions from the guluronic acids with divalent cations (Ca^{2+} , Sr^{2+} , or Ba^{2+}). This results in a chain–chain association to form the “egg box model”.

There are some disadvantages of involving alginate polymer to form capsules. Alginate capsules are very porous and sensitive to acidic environment (Gouin, 2004; Mortazavian et al., 2008), which is not compatible for bacteria preservation and resistance of the microparticles under stomach conditions.

2.1.2. κ -Carrageenan

κ -carrageenan is a neutral polysaccharide extracted from marine macroalgae, commonly used in food industry. There are three types of carrageenan (κ -, ι - and λ -) commonly used in foods and exhibit different gelation properties: κ -carrageenan forms rigid, brittle gels; ι -carrageenan produces softer, elastic and cohesive gels; while λ -carrageenan does not form gels (Fig. 3).

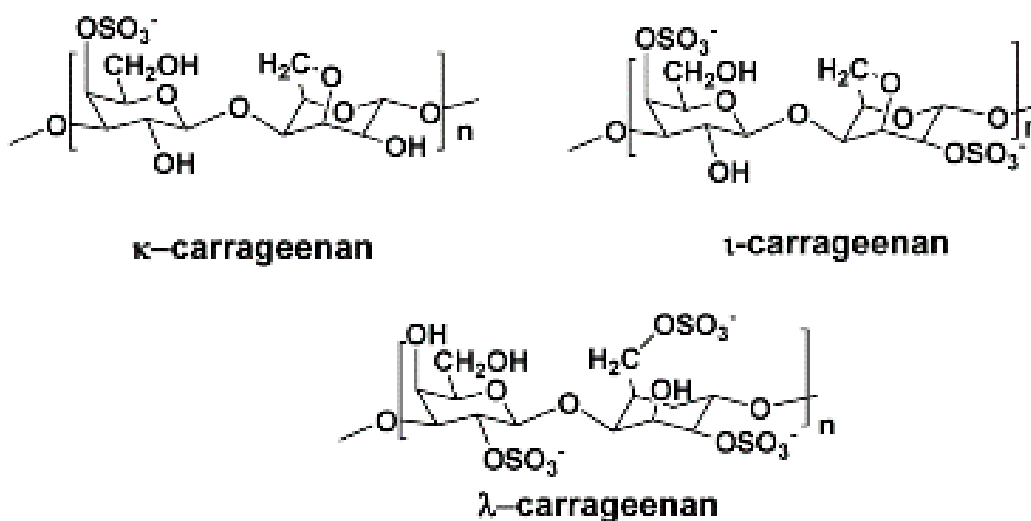


Figure 3: Carrageenan structure.

These differences can be attributed to differences in sulfate groups and anhydro-bridges (Burey, Bhandari, Howes, & Gidley, 2008). They can be used to form microcapsules by using different production techniques. Carrageenan formed gels through ionotropic gelation coupled to a cold mechanism, which involves helix formation upon cooling and crosslinking in the presence of K⁺ ions (in the form of KCl) to stabilize the gel and prevent swelling, and induce gelation.

However, KCl has been reported to have an inhibitory effect on some lactic acid bacteria. As an alternative to K^+ , NH_4^+ ions have been recommended and produce stronger gel capsules (Krasaekoopt et al., 2003).

High concentration of carrageenan such as 2-5% needs temperatures between 60-90°C for dissolution. Gelation is induced by temperatures changes. Probiotics are added into the polymer solution at 40-45°C and gelation occurs by cooling down to room temperature. Encapsulation of probiotic cells in κ -carrageenan beads keeps the bacteria in a viable state (Dinakar & Mistry, 1994) but gels produced are brittle and not able to withstand stresses (Chen & Chen, 2007).

2.1.3. Cellulose

Cellulose is a structural polysaccharide from plants that is available to use in the food industry after several physically or chemically modification.

Cellulose acetate phtalate (CAP)

This polymer is a derivate of cellulose (Fig 4) that is used with drugs for controlled drug release in the intestine (Mortazavian et al., 2008). CAP is insoluble in acid media ($pH \leq 5$) but soluble when the $pH \geq 6$. It provides a good protection for probiotic bacteria in simulated gastrointestinal (GI) conditions (Favaro-Trindade & Grosso, 2002), (Burgain et al., 2011). (Rao, Shiwnarain, & Maharaj, 1989) found that preparing an emulsion with starch and oil and adding CAP improved high viability of probiotics in simulated gastric environment.

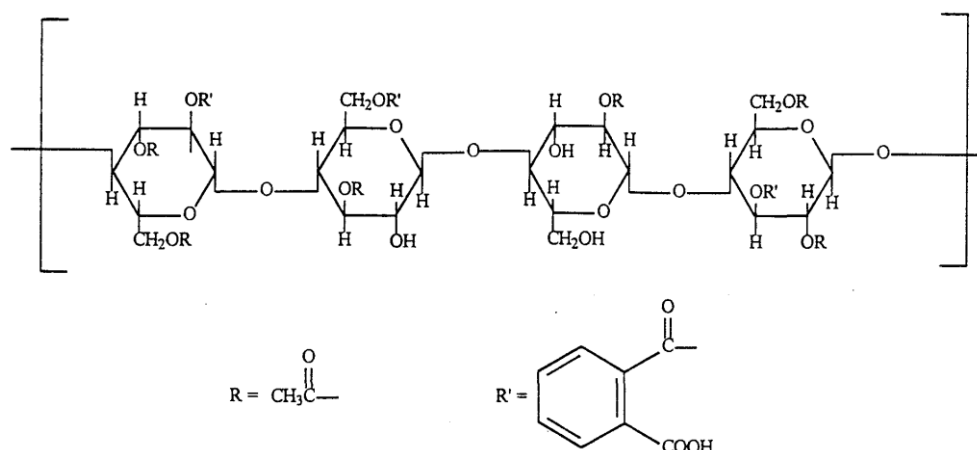


Figure 4: Cellulose acetate phtalate (CAP) structure

Sodium carboxymethyl cellulose

Sodium carboxymethyl cellulose (NaCMC) is a hydrophilic polyanionic cellulose that is commonly used as a food-grade biopolymer for building block for assembling polymers (Tripathy & Raichur, 2013). It consists of linked glucopyranose residues with varying levels of carboxymethyl substitution (Fig 5). CMC is not biodegradable and has been used as a hydrophobic polymer to produce hollow-shell particles with solid core (Gunduz, Ahmad, Stride, & Edirisinghe, 2013 ; Montes, Gordillo, Pereyra, & de la Ossa, 2011). It can be used with drugs and probiotics because of its resistance to gastric acid and intestinal solubility properties (Hubbe, Rojas, Lucia, & Sain, 2008).

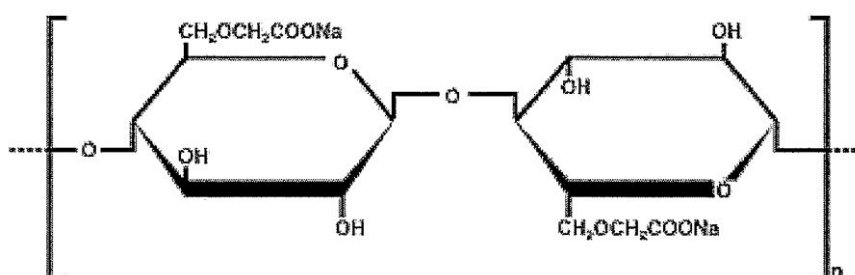


Figure 5: Sodium carboxymethyl cellulose (NaCMC) sturcture

2.1.4. Xanthan gum

Xanthan Gum is an extracellular anionic polysaccharide extracted from microbial source *Xanthomonas campestris*. It is a complex polysaccharide consisted of a primary chain of β -D-(1,4)-glucose backbone, which has a branching tri-saccharide side chain comprised of β -D-(1,2)-mannose, attached to β -D-(1,4)-glucuronic acid, and terminates in a β -D-mannose (Elçin, 1995), (Goddard & Gruber, 1999) (Fig. 6). Xanthan is soluble in cold water, and hydrates rapidly. It is considered to be basically non-gelling. The viscosity gradually reduces with increasing shear stress, thereafter returning to the stable over a wide range of pH (2-12) and temperature. The H-bonding and polymer chain entanglement formed network this is lead to high viscosity. Two chains may be aligned to form a double hemlix, providing a rather rigid configuration. The conversion between the ordered double helical conformation and the single more flexible extended chain may take place over hours between 40°C and 80°C (Nedovic et al., 2011). The extraordinary resistance to enzymatic degradation is attributed to the shielding of the backbone by side chain. Xanthan undergoes cryogelation (Giannouli & Morris, 2003).

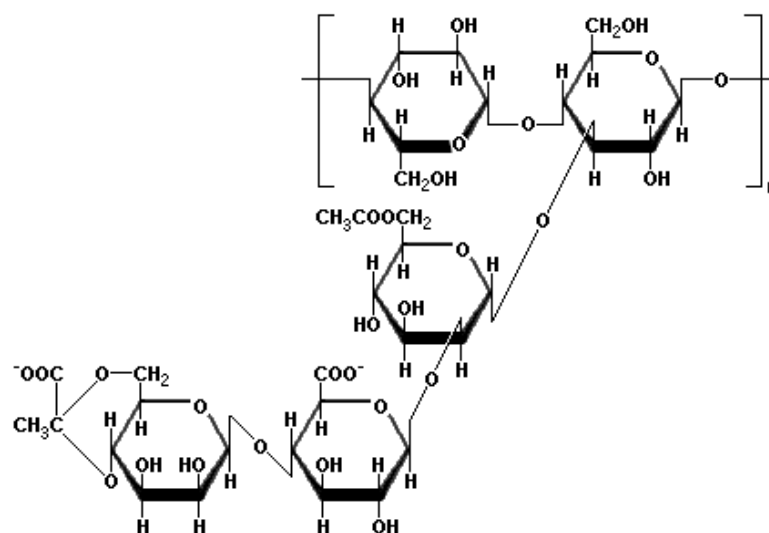


Figure 6: Xanthan gum structure

2.1.5. Chitosan

Chitosan is a linear polysaccharide with positive charge, which is obtained by extracted chitin from crustacean shells then deacetylated. Chitosan is a hydrophilic, cationic and crystalline polymer that demonstrates film forming ability and gelation characteristics. It is composed of glucosamine units, which can polymerize by cross-linking in the presence of anions and polyanions (Fig. 7). Chitosan is preferably used as a coating than capsules because it is not efficient for increasing cell viability by simple encapsulation (Mortazavian et al., 2008). Chitosan capsules are efficient with hydrophilic macromolecules through electrostatic interaction or hydrogen bonding. However, chitosan seems to have inhibitory effects on LAB (Groboillot, Champagne, Darling, Poncelet, & Neufeld, 1993).

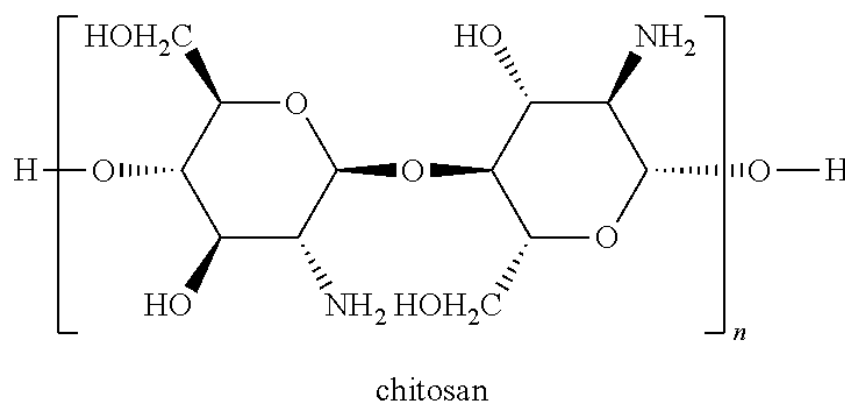


Figure 7: Chitosan structure

2.1.6. Pectin

Pectin is a heteropolysacchride that enhances cellulose structures in plant cell walls. The backbone of pectin contains regions of galacturonic acid residues that can be methoxylated. Natural pectin typically has a degree of esterification of 70 to 80%, but this may be diverse by changing extraction and production conditions. Pectin gelation and structure forming characteristics are due to the degree of esterification (DE) and the arrangement of the methyl

groups over the pectin molecule (Fig. 8). Gelation of high methoxyl pectin (HMP) requires a pH about 3 and a insoluble solids, while gelation of low methoxyl pectin (LMP) requires the presence of a controlled amount of calcium ions and need neither sugar nor acid (Joye & McClements, 2014). Pectin form rigid gels by reacting with calcium salts or multivalent cations, which crosslink the galacturonic acids of the main polymer chains. Calcium pectinate ‘eggbox’ structure is obtained by combination of the carboxylic acid groups of the pectin molecules and the calcium ions. (Jain, Gupta, Jain, & others, 2007); (L. Liu, Fishman, Kost, & Hicks, 2003); (Tiwari, Ramteke, Dahima, & Shukla, 2011). Biopolymer particles can be produced by combination of pectin with other polymers or cations. Its degradation rate can also be modified by chemical-modification (de Vos, Faas, Spasojevic, & Sikkema, 2010). Pectin is degradable by bacteria. Therefore, it can be used as gelling agent in food, in medicines and as a source of dietary fiber, because it remains intact in the stomach and the small intestine.

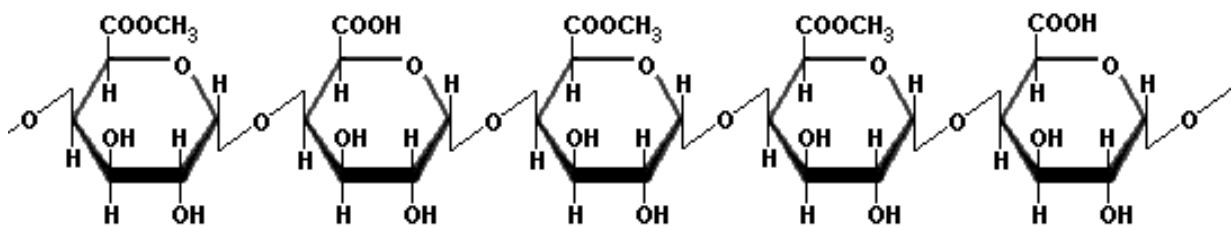


Figure 8: Pectin structure

2.1.7. Dextran

Dextran is an extracellular bacterial polymer of D-glucopyranose, hydrophilic neutral polysaccharide, with predominantly α -(1 \rightarrow 6) linkage in the main chain and a variable amount of α -(1 \rightarrow 2), α -(1 \rightarrow 3), α -(1 \rightarrow 4) branched linkages (Fig. 9). Dextran is one of the polysaccharide promising target for modification, because it has a large number of hydroxyl groups (Suarez, Grover, Braden, Christman, & Almutairi, 2013); (H. Wang et al., 2012). Dextran are well soluble in water, but is modified to change the solubility pattern, that has implications when

forming biopolymer particles by antisolvent precipitation (Broaders, Cohen, Beaudette, Bachelder, & Fréchet, 2009). Dextran is largely used as biomaterial in the field of polysaccharide polymers, because the required chemical modifications by hydroxyl are low cost (Hu & Jing, 2009); (Kuen Yong Lee, Jeong, Kang, Lee, & Park, 2009). Dextran is rapidly degraded by dextranases produced in the gut.

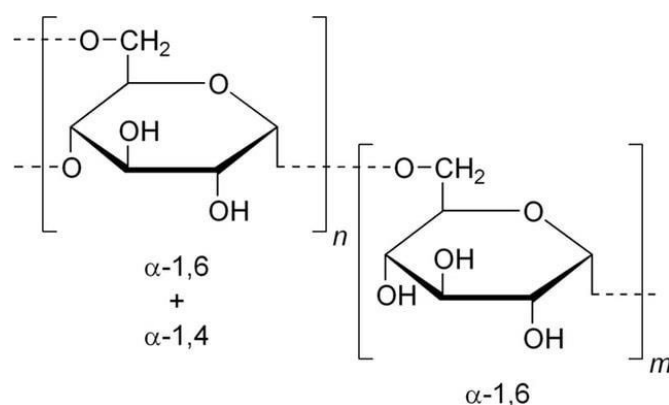


Figure 9: Dextran structure

2.1.8. Gellan gum

Gellan gum is a microbial polysaccharide derived from *Pseudomonas elodea* which is constituted of a repeating unit of tetrasaccharides composed of two glucose units, one glucuronic acid and one rhamnose (M.-J. Chen & Chen, 2007) (Fig. 10). Gellan is not easily degraded by enzymes, and stable over wide pH range; therefore it is used in the food industry (Nag, Han, & Singh, 2011), (Yang, Xia, Tan, & Zhang, 2013). Gellan gum particles are useful for colonic delivery of active compounds because, it can be degraded by galactomannanases in colonic fluids (Yang et al., 2013). To forming delivery system, there are two types of gellan gum : the highly acetylated (native) and the deacetylated forms (Singh & Kim, 2005).

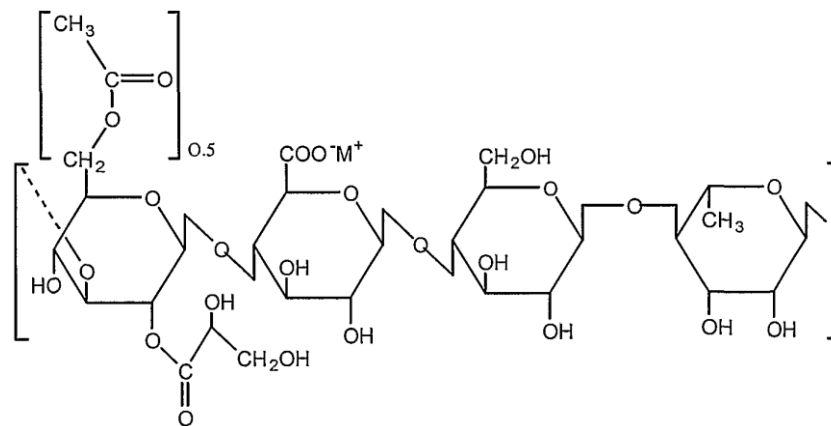


Figure 10: Gellan gum structure

2.1.9. Starch

Starch consists of a mixture of two neutral polysaccharides (linear amylose and branched amylopectin). It is insoluble in cold water, ethanol, and most common solvents. Starch consists mainly in linear amylose, a linear polymer of D-glucopyranose joined by α -1-4 glucosidic bond and amylopectin, a branched polymer of glucose joined by α -1-4 glucosidic bond and α -1-6 glycosidic bond for ramification (Fuentes-Zaragoza, Riquelme-Navarrete, Sánchez-Zapata, & Pérez-Álvarez, 2010) (Fig 11). The starch is not digested by amylases in the small intestine (Singh, Dartois, & Kaur, 2010); (Anal & Singh, 2007) and releases bacterial cells in the large intestine (Mortazavian et al., 2008). It improves probiotic delivery in a viable and a metabolically active state to the intestine, because it has an ideal surface for the adherence of the probiotic cells (Anal & Singh, 2007; Crittenden et al., 2001).

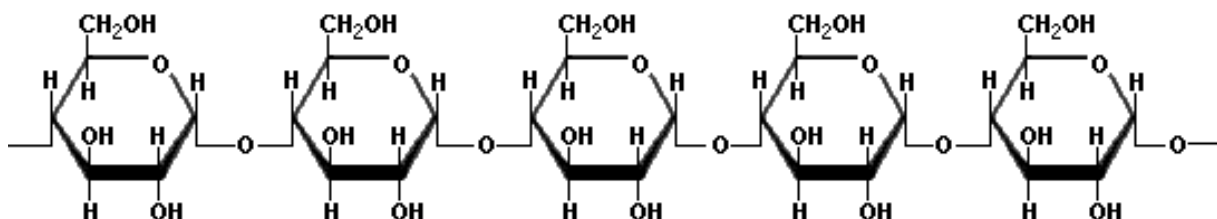


Figure 11: Starch structure.

2.2. Proteins

2.2.1. Collagen

Collagen is the major component of mammalian connective tissue. It is found in high concentrations in tendon, skin, bone, cartilage and, ligament. The collagen protein is composed of a triple helix, which generally consists of two identical chains ($\alpha 1$) and an additional chain that differs slightly in its chemical composition ($\alpha 2$) (Fig. 13). Due to its biocompatibility, biodegradability, abundance in nature, and natural ability to bind cells, it has been used in cell immobilization. Human like collagen (HLC) is produced by recombinant *Escherichia coli* BL21 containing human like collagen cDNA.

Collagen may be gelled utilizing changes in pH, allowing cell encapsulation in a minimally traumatic manner (Rosenblatt, Devereux, & Wallace, 1994),(Senuma et al., 2000). It may be processed into fibers and macroporous scaffolds (Chevallay & Herbage, 2000),(Roche, Ronziere, Herbage, & Freyria, 2000), while (Su et al., 2011) prepared microspheres using alginate and HCL by electrostatic droplet generation. The results showed encapsulation probiotic bacteria improving tolerance of in simulated gastric juice than free probiotic bacteria.

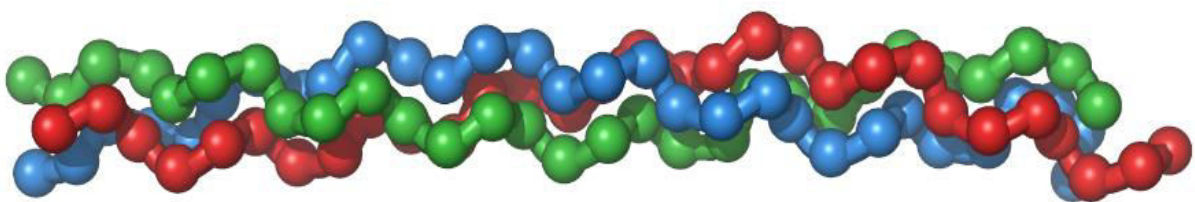


Figure 12: collagen triple helix (from Wikipedia)

2.2.2. Gelatin

Gelatin is a protein derived from collagen by partial hydrolysis. It contains between 300 and 4,000 amino acids (Fig. 13). Gelatin is soluble in most polar solvents and forms a solution of

high viscosity in water. Gelatin gels formed on cooling of solutions with concentrations above about 1%wt. The form of gels depends on the quality of the gelatin and the pH; they are clear, elastic, transparent, and thermo-reversible; it dissolves again on 35-40°C (Zuidam & Nedovic, 2010). Gelatin has been used for prepare microcapsules (probiotic bacteria) alone or with other compounds. When pH of gelatin is below the isoelectric point, the net charge of gelatin is positive then formed and a strong interaction with the negatively charged materials (Krasaekoopt et al., 2003), (Anal & Singh, 2007). Higher concentrations of gelatin produce strong capsules that are tolerant against crackling and breaking. Gellan gum and gelatin mixing has been used for the encapsulation of *Lactobacillus lactis* ssp. *cremoris* (Hyndman et al., 1993).

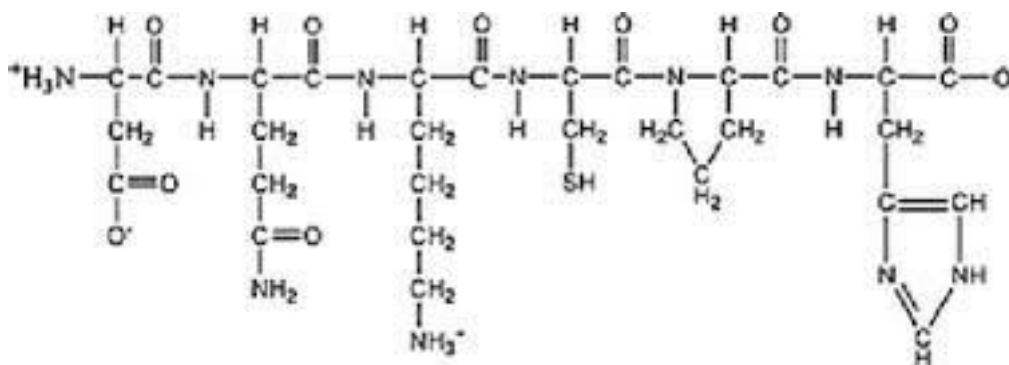


Figure 13: Gelatin structure

2.2.3. Milk proteins

Caseins

Caseins are the most prevalent phosphoproteins in milk and are extremely heat-stable proteins. They are obtained by destabilizing skim milk micelles by various processes. The main casein types are presented in Fig. 14. The products obtained are mineral acid casein, lactic acid casein, and rennet casein. The microcapsules can be prepared by using milk protein solution and enzyme rennet. Aggregation of the casein occurs by using rennet enzyme cleaving the k-casein molecule (Heidebach, Först, & Kulozik, 2012). Non-covalent cross-links are then gradually formed

between chains to form a final gel above 18° C (Bansal, Fox, & McSweeney, 2007). They are able to encapsulate probiotics, without significant cells loss during encapsulation process. Caseins can protect the bacteria in capsules during incubation under simulated gastric conditions. This technique is suitable for application with probiotic in food.

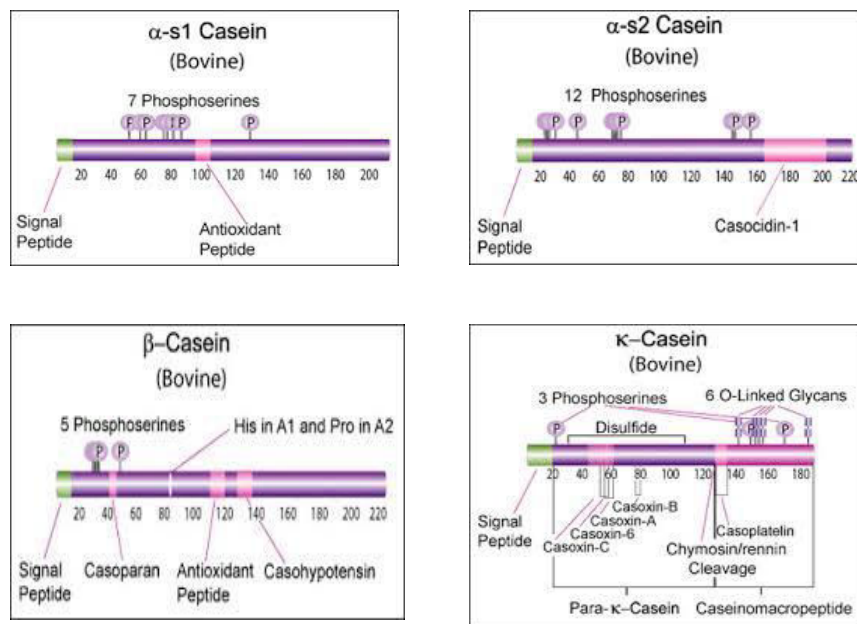


Figure 14: Caseins structure

Whey protein

Whey is a by-product of cheese or casein production. Whey protein include α -lactalbumin, β -lactooglobulin, immunoglobulins, and serumalbumin but also various minor proteins (Fig.15). Whey protein can be gelled by two different way, such as heat induced gelation (heating above the thermal denaturation temperature under appropriate pH and ionic strength conditions) or cold induced gelation (addition of calcium to a mixture using both extrusion and phase separation methods). Whey proteins gel are produced using various whey proteins, such as β -lactooglobulin (Jones, Decker, & McClements, 2010), (Jones & McClements, 2011), and lactoferrin (Bengoechea, Jones, Guerrero, & McClements, 2011).

Whey protein particles have been produced for encapsulation of probiotics with a good protection (Huq, Khan, Khan, Riedl, & Lacroix, 2013), (Doherty et al., 2011).

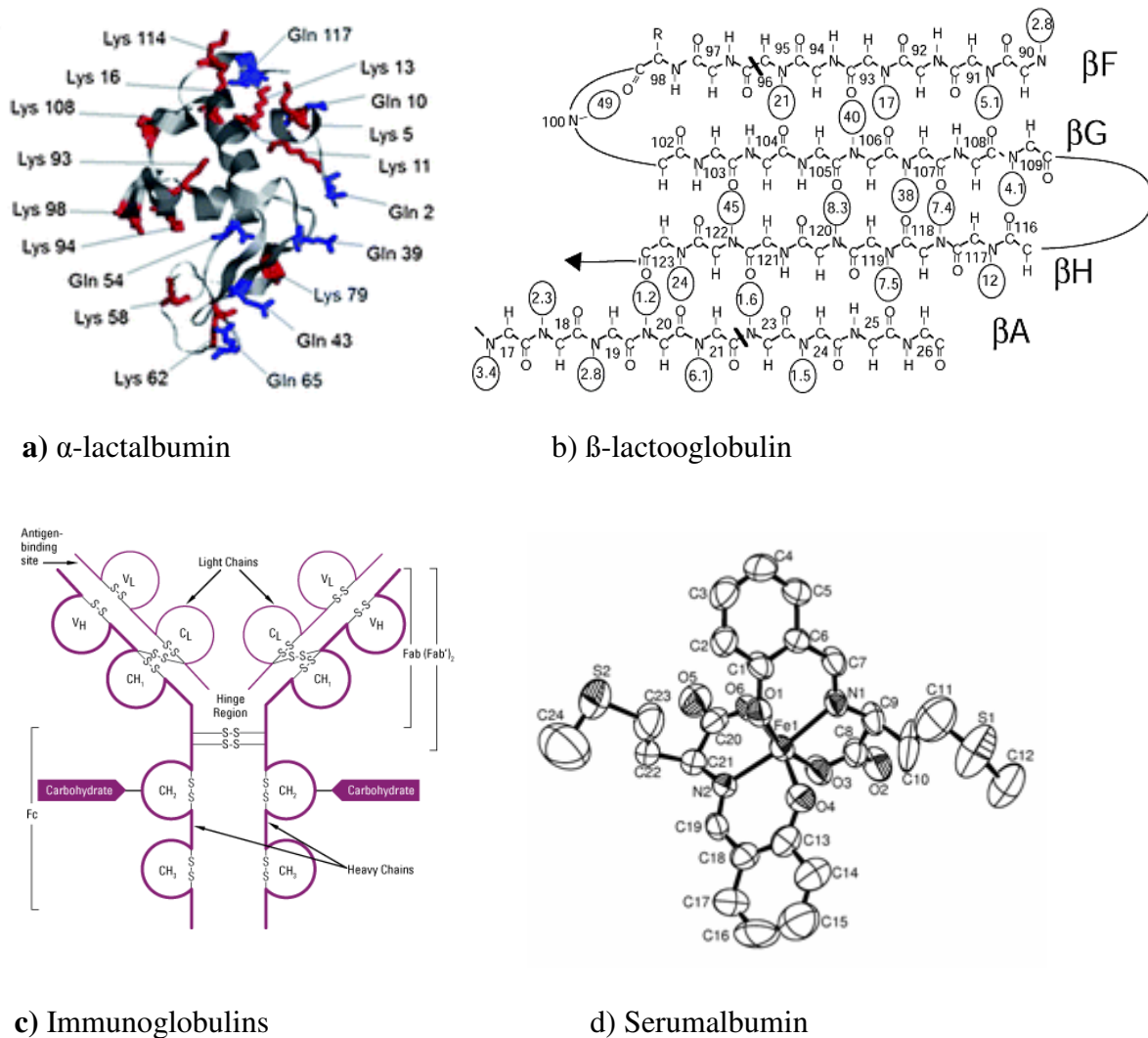


Figure 15: Whey Protein structure

2.2.4. Plant-derived proteins.

Protein derived from plants provide some advantages over animal proteins to form a biopolymers molecules: reduced risk of contamination and infection, cheaper, usable in vegetarian food products. (Regier, Taylor, Borczyk, Yang, & Pannier, 2012) –(L. Chen & Subirade, 2009). Among all vegetal proteins, hydrophobic cereal proteins are largely used to produce biopolymer particles by various methods. These particles are shown suitable for

encapsulation of active ingredients (A. Patel, Hu, Tiwari, & Velikov, 2010a),(Ezpeleta et al., 1996),(Duclairoir, Orecchioni, Depraetere, & Nakache, 2002). because they are water insoluble, biodegradable and biocompatible. Emulsifier-stabilized protein particles may be used to improve the chemical stability of encapsulated active ingredients (Podaralla & Perumal, 2012) ,(A. Patel et al., 2010a),(A. R. Patel, Heussen, Dorst, Hazekamp, & Velikov, 2013). because it have good physical stability over a wide range of pH conditions (A. Patel et al., 2010). Other plant proteins that have recently been shown to be suitable for producing biopolymer particles include pea leguminous (Pierucci, Andrade, Farina, Pedrosa, & Rocha-Leão, 2007) and soy protein isolate (Liu & Tang, 2013). Soy protein isolate (SPI) contains an abundant of mixture hydrophilic globular proteins, cheap, and renewable source.

Chickpea proteins showed also good functional attributes. Two salt-soluble globulin-type proteins dominate legumin and vicilin. (J. Wang, Korber, Low, & Nickerson, 2014). Chickpea protein coupled with alginate were designed to serve as a suitable probiotic carrier intended for food applications (Klemmer, Korber, Low, & Nickerson, 2011). These capsules were able to protect *B. adolescentis* within simulated gastric juice and simulated intestinal fluids.

Table 1: comparison of various carbohydrates used for bacteria encapsulation in the literature

Carbohydrates	Advantages	Disadvantages	Interest with probiotics	References
Alginate	<ul style="list-style-type: none"> - Simplicity. - Non toxicity. - Biocompatibility. - Low cost. 	<ul style="list-style-type: none"> - Alginate beads are sensitive to the acidic environment. - Not compatible for the resistance of the microparticles in the stomach conditions. - Very porous. 	+	<p>(Krasaekoopt et al., 2003)</p> <p>(Mortazavian et al., 2008)</p>
κ -Carrageenan	<ul style="list-style-type: none"> - Softer, elastic and cohesive gels. - K⁺ ions to stabilize the gel and prevent swelling 	<ul style="list-style-type: none"> - Brittle gels ; not able to withstand stresses. - KCl has been reported to have an inhibitory effect on some lactic acid bacteria. 	-	<p>(M.-J. Chen & Chen, 2007)</p> <p>(Burey et al., 2008)</p> <p>(Krasaekoopt et al., 2003)</p>
Cellulose	<p>a- Cellulose acetate phtalate (CAP)</p> <ul style="list-style-type: none"> - Insoluble in acid media (pH \leq5). - Good protection for microorganisms - Adding CAP with starch and oil improved viability of probiotics 	<p>a- Cellulose acetate phtalate (CAP)</p> <ul style="list-style-type: none"> - Soluble when the pH is \geq6. 	+	<p>(Favaro-Trindade & Grosso, 2002)</p> <p>(Burgain et al., 2011)</p> <p>(Rao et al., 1989)</p> <p>(Tripathy & Raichur, 2013)</p>
			+	

	<p>b- Sodium carboxymethyl cellulose (NaCMC).</p> <ul style="list-style-type: none"> - Ethylcellulose is not biodegradable and has been used as a hydrophobic polymer to produce hollow-shell particles and particles with a solid core. 	<p>b- Sodium carboxymethyl cellulose (NaCMC).</p> <ul style="list-style-type: none"> - High hydrophilicity. 		<p>(Gunduz et al., 2013)</p> <p>(Montes, Gordillo, Pereyra, & de la Ossa, 2011)</p> <p>(Hubbe et al., 2008)</p>
Xanthan Gum	<ul style="list-style-type: none"> - Stable over a wide range of pH (2-12) and temperature. - Resistance to enzymatic degradation. 	<ul style="list-style-type: none"> - Soluble in cold water, hydrates rapidly. It is considered to be basically non-gelling 	+	<p>(Elçin, 1995)</p> <p>(Goddard & Gruber, 1999)</p> <p>(Nedovic et al., 2011)</p> <p>(Giannouli & Morris, 2003)</p>
Chitosan	<ul style="list-style-type: none"> - Useful for encapsulation of hydrophilic macromolecules. 	<ul style="list-style-type: none"> - Poor efficiency for increasing cell viability by encapsulation and it is preferable to use as a coat but not as a capsule. - Inhibitory effects on LAB. 	-	<p>(Mortazavian et al., 2008)</p> <p>(Groboillot et al., 1993)</p>
Pectin	<ul style="list-style-type: none"> - Can be used to form biopolymer particles in combination with other polymers. - Is used as gelling agent in food, in medicines and as a source of dietary fiber. 	<ul style="list-style-type: none"> - High degradation rate. 	+	<p>(Joye & McClements, 2014)</p> <p>(Jain et al., 2007)</p> <p>(L. Liu et al., 2003)</p> <p>(Tiwari et al., 2011)</p> <p>(de Vos et al., 2010)</p>

-It remains intact in the stomach and the small intestine.

Dextran	<ul style="list-style-type: none"> - Utilization in the field of polysaccharide polymer conjugates for biomaterials. - Is rapidly degraded by dextranases produced in the gut. 	<ul style="list-style-type: none"> - Soluble in water. - Degradation rapidly and acid-sensitivity 	+	<p>(Suarez et al., 2013)</p> <p>(H. Wang et al., 2012)</p> <p>(Hu & Jing, 2009)</p> <p>(Kuen Yong Lee et al., 2009)</p>
Gellan gum	<ul style="list-style-type: none"> - Not easily degraded by enzymes. - Stable over widely pH range. - Useful for colonic delivery of active compounds. 		+	<p>(M.-J. Chen & Chen, 2007)</p> <p>(Yang et al., 2013)</p> <p>(B. N. Singh & Kim, 2005).</p>
Starch	<ul style="list-style-type: none"> - Good enteric delivery characteristic that is a better release of the bacterial cells in the large-intestine. - Ideal surface for the adherence of the probiotic cells to the starch granules. - Improving probiotic delivery in a viable and a metabolically active state to the intestine. 	<ul style="list-style-type: none"> - Poor solubility. - High surface tension. 	+	<p>(Fuentes-Zaragoza et al., 2010)</p> <p>(J. Singh et al., 2010)</p> <p>(Anal & Singh, 2007)</p> <p>(Mortazavian et al., 2008)</p> <p>(Crittenden et al., 2001)</p>

Table 2: comparison of various proteins used for bacteria encapsulation in the literature

Protein	Advantages	Disadvantages	Interest with probiotics	References
Gelatin	<ul style="list-style-type: none"> - Clear, elastic, transparent, and thermo-reversible gels. - used for probiotic encapsulation. 	<ul style="list-style-type: none"> - High deformation of capsules. - Low the values of viscoelastic parameters. 	+	(Zuidam & Nedovic, 2010) (Krasaekoopt et al., 2003) (Hyndman et al., 1993)
Collagen	<ul style="list-style-type: none"> - Used in cell immobilization due to its biocompatibility, biodegradability, abundance in nature, and natural ability to bind cells. - can form a high-water-content hydrogel composite. 	<ul style="list-style-type: none"> - High cost to purify, natural variability of isolated collagen. - A lot of pores formed possibly due to sublimation of water 	+	(Rosenblatt et al., 1994) (Senuma et al., 2000) (Chevallay & Herbage, 2000) (Roche et al., 2000) (C. H. Lee et al., 2001) (Yann (Juncosa-Melvin et al., 2007)
Milk Proteins	a- Caseins <ul style="list-style-type: none"> - These able to encapsulate probiotics, without significant loss of cells during the encapsulation process. - Protect bacteria in capsules during incubation under simulated gastric conditions at low pH. 	<ul style="list-style-type: none"> - Irregular shaped particles. - The capsules should be low 100 μm as desirable to avoid negative sensorial impacts for microcapsules in food. 	+	(Heidebach, Först, & Kulozik, 2009) (Bansal et al., 2007)

b- Whey Protein

- Whey protein particles have been produced for encapsulation of probiotics

+ (Jones et al., 2010)
 (Jones & McClements, 2011)
 (Bengoechea et al., 2011)
 (Gülseren et al., 2012)

Plant-derived proteins.

- Proteins plants provide some advantages over animal proteins to form a biopolymers molecules since the risk of contamination and infection is reduced

- (Podaralla & Perumal, 2012)
 (A. R. Patel et al., 2013).
 (A. Patel, Hu, Tiwari, & Velikov, 2010b)
 (F. Liu & Tang, 2013)

- Cheap.

- Can be used in vegetarian products.

- Water insoluble, biodegradable and biocompatible.

- Good physical stability over a range of pH conditions.

+ (J. Wang et al., 2014)
 (Klemmer et al., 2011)

Chickpea Protein

- Good functional attributes and nutritional importance.

- Good protection to probiotic bacteria

3. Microencapsulation methods

There are several encapsulation techniques. Before selecting one of them, people must take into consideration the following point (N.J. Zuidam & Nedovic, 2010):

- i) What conditions that affect the viability of probiotics?
- (ii) Which processing conditions are used during food production?
- (iii) What will be the storage conditions of the food product containing the Microcapsules?
- (iv) Which particle size is needed to incorporate in the food product?
- (v) What are the mechanisms of release?
- (vi) What are the cost constraints?

Different technologies are used to produce microcapsules according to the answers of the above questions. They are presented below.

3.1. Methods to produce dry capsules

3.1.1. Spray Drying

Spray-drying is one of the oldest process and the most widely used in microencapsulation technique in the food industry sector. It is an economical and flexible operation. The process involves the atomization of a suspension of microbial cells in polymeric solution in a chamber supplied with hot air. This lead to solvent evaporation. The dried particles are then separated by a filter or cyclone (M.-J. Chen, Chen, & Kuo, 2007); (de Vos et al., 2010); (K. Kailasapathy, 2009); (Zhao, Sun, Torley, Wang, & Niu, 2007). There are different parameters to optimize spray-drying such as air flow, feed rate, feed temperature, inlet air temperature, and outlet air temperature (Vega & Roos, 2006); (O’Riordan, Andrews, Buckle, & Conway, 2001).

There are many advantages for spray-dried process : it can be operated on a continuous basis, it is rapid and relatively low cost, it can be applied on a large scale suitable for industrial

applications (Brun-Graeppi, Richard, Bessodes, Scherman, & Merten, 2011); (Gouin, 2004). Spray-drying disadvantages are linked to the “high” temperature used to dry, which is not suitable for probiotic bacteria (Favaro-Trindade & Grosso, 2002); (Ananta, Volkert, & Knorr, 2005);(Oliveira, Moretti, Boschini, Baliero, Freitas, & Favaro-Trindade, 2007). The compatibility of bacteria strain and type of encapsulating polymers have to be controlled to allow bacteria survival during spray-drying process as well as during storage (Desmond, Ross, O’Callaghan, Fitzgerald, & Stanton, 2002).

3.1.2. Freeze-drying

Freeze-drying has been largely used to producing probiotic powders. During the process, the solvent or the suspension medium is frozen then sublimed (Santivarangkna, Kulozik, & Foerst, 2007; Solanki et al., 2013). The freeze-drying process is divided into three stages; freezing, primary drying, and second drying. The freeze-drying processing condition is milder than spray-drying and higher probiotic survival rates are typically achieved (Wang et al., 2014). Freeze-drying disadvantage is linked to crystal formation and stress condition due to high osmolarity that cause damages to cell membrane. To increase the viability of probiotics during dehydration, skim milk powder, whey protein, glucose, maltodextrin, trehalose are added to the drying media before freeze-drying to act as cryoprotectants (Mimoza Basholli-Salihi, 2014). Cryoprotectants reduce the osmotic difference between the internal and external environments by accumulation within the cells (Kets, Teunissen, & de Bont, 1996).

3.1.3. Spray freeze-drying

Spray-freeze-drying technique combines processing steps that are common to freeze-drying and spray-drying. Probiotic cells are in a solution, which is atomized into a cold vapor phase of a

cryogenic liquid such as liquid nitrogen. The microcapsules formed by dispersion of frozen droplets then dried in a freeze dryer (Amin, Thakur, Jain, 2013); (H. Wang et al., 2012); (de Vos et al., 2010); (K. Kailasapathy, 2009); (Semyonov et al., 2010). The main advantages of spray-freeze-drying techniques are: controlled size, higher surface area for capsules in spray freeze dried. This technique has nevertheless some disadvantages including the high use of energy, the long processing time and the cost which is 30–50 times more expensive than spray-drying (Zuidam & Nedovic, 2010). In some studies, using polysaccharides contributes to reduce cells mobility in the glassy state matrix, that act as a protective excipient and improve cell viability during freezing (Semyonov et al., 2010).

3.1.4. Spray-chilling / Spray-cooling

Spray-chilling, spray-cooling, spray-congealing are similar processes as spray-drying, but no water is evaporated and the air used is cold, which enables particle solidification. The microcapsules are quickly formed when the matrix containing the bioactive compound is into contact with the cold air (Champagne & Fustier, 2007). The spray-chilling mainly uses a molten lipid matrix as carrier. Spray-chilling disadvantages are that the capacity of encapsulation is low and the release of core material is observed during storage (Sato & Ueno, 2005). Spray-chilling technique is a cheaper encapsulation technology that has potential for industrial scale manufacture (Gouin, 2004). This technology could generate smaller beads, which be desirable in food processing. (Pedroso, Thomazini, Heinemann, & Favaro-Trindade, 2012) used spray-chilling technology to prepare microencapsulate *Bifidobacterium lactis* and *Lactobacillus acidophilus* using as wall materials with palm and palm kernel. The solid lipid microparticles provided an effective protection for probiotics against gastric and intestinal fluids.

3.1.5. Fluid bed

In the fluid bed process, a cell suspension is sprayed and dried on inert carriers. The fluid bed advantages are mainly the control over the temperature and the lower cost. The disadvantages are that this technology is difficult to master for long duration. This method can be used to produce multilayer coatings with two different fats (Champagne, Raymond, & Tompkins, 2010). Fluid bead is one of the most used encapsulation technologies commercially applied to probiotics. Some companies have developed commercial products, such as Probiocap® and Duaolac® (Burgain et al., 2011).

3.1.6. Impinging aerosol technology

Impinging aerosol technology is used two separate aerosols: One with the microbial suspension in alginate solution and the other one with calcium chloride. The mixture of alginate is injected from the top of a cylinder and on the mean time the calcium chloride is injected from the base to produce alginate microcapsules (Sohail, Turner, Coombes, Bostrom, & Bhandari, 2011). The impinging aerosol technology advantages are: it is suitable for encapsulating heat labile and solvent sensitive materials, it provides a large volume production, the capsules could be spray or freeze-dried in a later stage. The microcapsules diameter of 2 mm was obtained and offered high protection to *L. rhamnosus* GG in gastic acid and bile (Sohail et al., 2011).

3.1.7. Electrospinning

This technique is a combination between two techniques namely electrospray and spinning. In this technique, a high electric field is applied to a fluid which comes out from the tip of a die that acts as one of the electrodes. This leads to droplet deformation and finally to the ejection of a charged jet from the tip towards the counter electrode leading to the formation of

continuous capsules (cylinder). The main advantage of electrospinning technique is that capsules are very thin with large surface areas “few nanometers” (Agarwal, Wendorff, & Greiner, 2008). (López-Rubio, Sanchez, Wilkanowicz, Sanz, & Lagaron, 2012) compared two type of electrospinning microcapsules, encapsulated probiotics in a protein based matrix (whey protein concentrate) and in a carbohydrate based matrix (pullulan). Whey protein microcapsules proved a greater cell viability when compared to pullulan structures.

3.2. Methods to produce humid capsules

3.2.1. Emulsification and ionic gelation

Emulsification is a technique to encapsulate alive probiotics and uses different polysaccharides as encapsulating materials such as alginate, K-carrageenan, gellan-gum, xanthan, or pectin. For encapsulation in an emulsion, an emulsifier and/or a surfactant are needed. A solidifying agent is then added to the emulsion (Chen & Chen, 2007); (Kailasapathy, 2009); (de Vos et al., 2010). This coupled technique gives a high survival rate of the bacteria but it provides capsules of large sizes and different shapes. The gel beads can be coated by a second polymer to provide better protection to the cell and improve organoleptic properties (K. Kailasapathy, 2009).

3.2.2. Emulsification and enzymatic gelation

This technique uses milk proteins with probiotics that are encapsulated by means of an enzymatic induced gelation; milk proteins have high gelation properties and offer a good protection for probiotics (Heidebach et al., 2009), (Heidebach et al., 2012). This method produces spherical particles, insoluble in water. (Heidebach et al., 2009) detailed an example of rennet gelation to prepare microcapsules. This technique permitted to use of alginate, K-

carrageenan, gellan-gum or xanthan for capsule coating, even they are not allowed for use in dairy products in some countries (Picot & Lacroix, 2003).

3.2.3. Emulsification and interfacial polymerization

Interfacial polymerization is an alternative technique that is performed in one-step. The technique requires the formation of an emulsion. The discontinuous phase contains an aqueous suspension of probiotic cells and the continuous phase is an organic solvent. To begin the polymerization reaction, a biocompatible agent is added. The microcapsules obtained are thin and have a strong membrane (Kaila Kailasapathy, 2002). Interfacial polymerization of microcapsules is used to improve the productivity in fermentation (Yáñez-Fernández, Ramos-Ramírez, & Salazar-Montoya, 2007).

3.2.4. Extrusion

Extrusion technique is the most popular method for humid microcapsules production (Green, Gill, Khan, & Vulfson, 1996), (Koyama & Seki, 2004), (Özer, Uzun, & Kirmaci, 2008). It involves the preparation of a hydrocolloid solution, mixed with the microbial cells and the extrusion of the cell suspension through a needle, then dropped into the solution of a cross-linking agent (Heidebach et al., 2012). Gelation occurs by a combination between the polymer and the cross-linking agent. The advantages of this technique are the simplicity of its operation, the lower cost, and the operational conditions suitable for probiotic bacteria viability (de Vos et al., 2010). The main disadvantage for this technique is that the microcapsules are larger than 500 μm (Reis, Neufeld, Vilela, Ribeiro, & Veiga, 2006). In addition, rapid cross-linking between droplet polymers solution and cross link agent led to rapid hardening of microcapsules surfaces that delay the movement of cross-linking ions into the inner core (Liu et al., 2002).

3.2.5. Co-Extrusion

Co-Extrusion technology is based on a laminar liquid jet that is broken into equally sized droplets by a vibrating nozzle (Prüsse, Bilancetti, Bučko, Bugarski, Bukowski, Gemeiner, Lewińska, Manojlovic, Massart, Nastruzzi, Nedovic, et al., 2008), (Del Gaudio, Colombo, Colombo, Russo, & Sonvico, 2005). The droplets are then gelled in a cross-linking solution. The diameter of microcapsules is controlled by two main factors, whaic are the flow rate and polymer solution viscosity (Del Gaudio et al., 2005).

(Graff, Hussain, Chaumeil, & Charrueau, 2008) encapsulated *Saccharomyces boulardii* using a laminar jet break up technique. Microcapsules were obtained by coating with chitosan solution and significantly reduced the degradation of yeast cells in the gastrointestinal tract. (Huang, Wu, & Lee, 2010) obtained microcapsules with two concentrations of alginate solution that were introduced separately into the inner and outer chambers of the coaxial nozzle. The polymer droplets were cross-linked in calcium chloride solution. Adjusting the concentrations of the shell and core materials provided a high control on the size of alginate microspheres and on releasing of the microbial cells from the microspheres.

3.2.6. Coacervation

This technique can be used to encapsulate flavor oils, preservatives, enzymes as well as microbial cells (John, Tyagi, Brar, Surampalli, & Prévost, 2011); (Oliveira, Moretti, Boschini, Baliero, Freitas, Freitas, et al., 2007); (Oliveira, Moretti, Boschini, Baliero, Freitas, & Favaro-Trindade, 2007). This technique uses specific pH, temperature and composition of the solution to separate one or more incompatible polymers from an initial coating solution. The incompatible polymers are added to the coating polymer solution and the dispersion is stirred. The separation of incompatible polymer and deposition of dense coacervate phase surrounding the core material to form microcapsules occur as a result for changes in the physical parameters

(Gouin, 2004); (John et al., 2011); (N. Nihant, Grandfils, Jérôme, & Teyssié, 1995); (Oliveira, Moretti, Boschini, Baliero, Freitas, Freitas, et al., 2007) ; (Oliveira, Moretti, Boschini, Baliero, Freitas, & Favaro-Trindade, 2007). The most important factors for the coacervation technique are the volume of dispersed phase, the ratio of incompatible polymer to coating polymer, the stirring rate of the dispersion and the core material to be encapsulated (N. Nihant et al., 1995). In the coacervation technique, the composition and viscosity for polymers solution in supernatant phases act on size distribution, surface morphology and internal porosity of the microcapsules (Nicole Nihant et al., 1994), (N. Nihant et al., 1995). (Oliveira, Moretti, Boschini, Baliero, Freitas, Freitas, et al., 2007) used the coacervation technique to encapsulate *B. lactis* (BI 01) and *L. acidophilus* (LAC 4) in a casein/pectin complex. This technology presented a good encapsulation capacity and a controlled liberation of core material from the microcapsules by mechanical stress, temperature and pH changes. So, the coacervation technique is a favorable with probiotic bacteria (Oliveira, Moretti, Boschini, Baliero, Freitas, Freitas, et al., 2007). The coacervation method disadvantage is that it cannot be used for producing very small microspheres (John et al., 2011).

Table 3: Encapsulation methods

Encapsulation methods	Advantages	Disadvantages	Valid with probiotic bacteria
Spray Drying	<ul style="list-style-type: none"> - It can be operated on a continuous basis. - It can be applied on a large scale. - It is suitable for industrial application. - It is rapid and relatively low cost. 	<ul style="list-style-type: none"> - The high temperature used in the process may not be suitable for encapsulating probiotic bacteria. 	-
Freeze Drying	<ul style="list-style-type: none"> - The freeze-drying processing condition is milder than spray-drying higher probiotic survival rates are typically achieved. 	<ul style="list-style-type: none"> - Freezing causes damage to the cell membrane because of crystal formation and imparts stress condition by high osmolarity. 	+
Methods to produce dry capsules	<ul style="list-style-type: none"> - Controlled size. 	<ul style="list-style-type: none"> - The use of high energy. 	
Spray Freeze Drying	<ul style="list-style-type: none"> - Larger specific surface area than spray-dried capsules. 	<ul style="list-style-type: none"> - The long processing time and the cost which is 30–50 times expensive than spray-drying. 	+
Spray chilling/ Spray cooling	<ul style="list-style-type: none"> - cheapest encapsulation technology. - potential of industrial scale manufacture. - generates smaller beads. 	<ul style="list-style-type: none"> - The spray chilling mainly uses a molten lipid matrix as carrier. The micro particles that are produced can present some disadvantages, which include a low encapsulation capacity and the expulsion of core material during storage. 	+

Methods to produce humid capsules	Fluid bed	<ul style="list-style-type: none"> - Good temperature control. - Low cost. - Easy scale-up. 	<ul style="list-style-type: none"> - Technology difficult to control for longer duration. 	+
	Impinging aerosol technology	<ul style="list-style-type: none"> - Suitable for encapsulating heat labile and solvent sensitive materials. - Large volume production capacity. 		+
	Electrospinning	<ul style="list-style-type: none"> - Production of very thin capsules. - Large surface areas. 		+
	Emulsification and ionic gelation	<ul style="list-style-type: none"> - High survival rate of the bacteria. - Gel beads can be coated by second polymer that provides more protection for bacteria. 	<ul style="list-style-type: none"> - Large size ranges and shapes. 	+
	Emulsification and enzymatic gelation	<ul style="list-style-type: none"> - Processing of water insoluble and spherical particles. - Use of coatings. 	<ul style="list-style-type: none"> - Coating microcapsules (enzymatic induced gelation) by alginate, K-carrageenan, gellan-gum or xanthan that are not allowed in dairy products in some countries. 	+
	Extrusion	<ul style="list-style-type: none"> - Simple operations. - Low cost. - Mild operational conditions ensuring high cell viability. 	<ul style="list-style-type: none"> - Inefficiency in producing microspheres smaller than 500 μm. - Less stable microspheres. 	+
	Co-Extrusion	<ul style="list-style-type: none"> - size-controlled microspheres. 		+

Coacervation

- Significant protection of the microorganisms in the gastrointestinal tract.
- High production rate.
- Good encapsulation capacity.
- Controlled liberation of core material from the microspheres by mechanical stress.

- This method may not be used for producing small microspheres.

+

4. Techniques for capsules characterization

4.1. Microcapsules size, morphology and stability

The particle size is often the most important characteristic of the capsules, and was measured by different type of microscopy or light scattering.

4.1.1. Microscopy

Optical and electron microscopies are used to measure the size of capsules, the surface topography, the thickness of membrane and, sometimes, the permeability of capsules membrane.

4.1.1.1. Conventional Optical Microscopy

This microscope is used to characterizing structures of capsules that are $\geq 0.2 \mu\text{m}$ as fixed by the wavelength of visible light, and the size of capsules can be measured (N.J. Zuidam & Nedovic, 2010).

4.1.1.2. Confocal Laser Scanning Microscopy (CLSM)

(CLSM) produce in-focus images of a fluorescent specimen by optical sectioning. CLSM provides a better spatial 3D image than electron microscopy and provides additional information such as the three-dimensional localization and quantification of the encapsulated phase. CLSM may allow definition of encapsulation rate without the need for any destruction, extraction and chemical assays (Lamprecht, Schäfer, & Lehr, 2000). CLSM could also provide the distribution of polymers and cross-linking ions (Strand, Mørch, Espevik, & Skjåk-Braek, 2003).

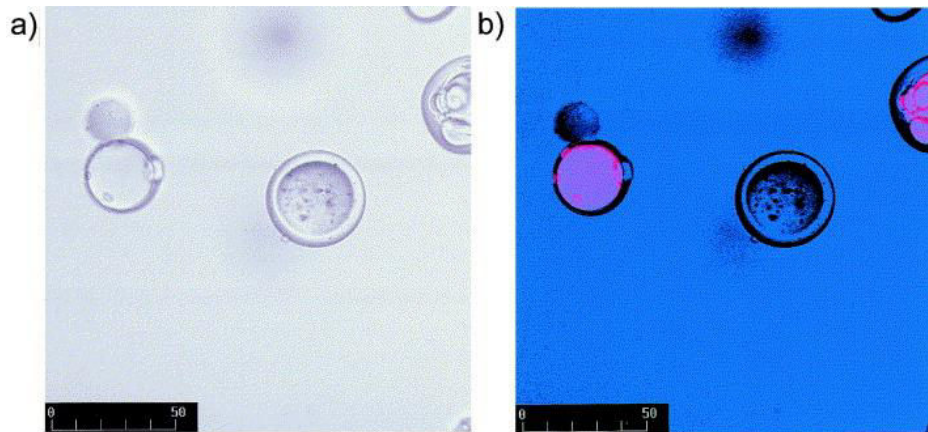


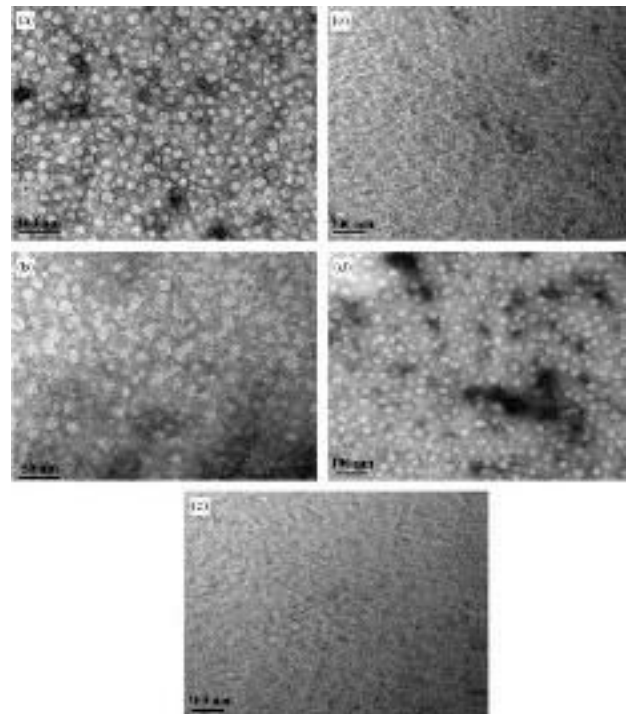
Figure 16: Visualization of microcapsules containing a Nile red stained oil phase by a light microscopy image (a) and by CLSM using the red fluorescence channel and transmitted light detection (b). The fluorescence signal allows the oil-containing and air-containing microcapsules to be unambiguously distinguished. Scale bar is shown in μm . (Lamprecht et al., 2000).

4.1.1.3. Transmission Electron Microscopy (TEM)

TEM is able of resolving structure with smaller dimension than optical microscopy (Dash, Murthy, Nath, & Chowdhury, 2010). TEM is used to measure the structures of very thin samples by passing electrons through them. TEM gives the morphology and shell thickness of encapsulates following fixation, dehydration, and sectioning (Chen & Subirade, 2005).

Figure 17:

- (a) TEM of CS- β lg nanoparticles (N-native) in simulated gastric fluid with pepsin for 0.5 h,
- (b) in simulated intestinal fluid with pancreatin for 10 h,
- (c) then degraded by chitosanase and lysozyme for 4 h.
- (d) TEM of chitosan nanoparticles in simulated intestinal fluid with pancreatin for 10 h,
- (e) and degraded by chitosanase and lysozyme for 4 h.



(Chiu et al., 2005) used TEM to analyze various liposome preparations (Fig.17). (Xu & Du, 2003) used TEM to examined diameter and spherical shape for capsules.

4.1.1.4. Scanning Electron Microscopy (SEM)

SEM provides information on surface characteristics, such as composition, shape and size (Mohsen Jahanshahi & Babaei, 2008), (Pierucci, Andrade, Farina, Pedrosa, & Rocha-Leão, 2007), (Montes, Gordillo, Pereyra, & Martínez de la Ossa, 2011). The samples must be frozen, dried or fractured and subsequently coated with metal compounds, which alter the representativeness of the sample. As an example, Rahimnejad, Jahanshahi, Najafpour (2006) (Fig. 18) determined nanoparticle size and distribution by SEM. The samples (protein (BSA) nanoparticle) were dipped into liquid nitrogen for 10 min, and then freeze-dried. The sample was fixed on the aluminum stub and coated with 20 nm of gold palladium. The shape of the nanoparticles were demonstrated spherical with sizes absolutely below 100 nm.

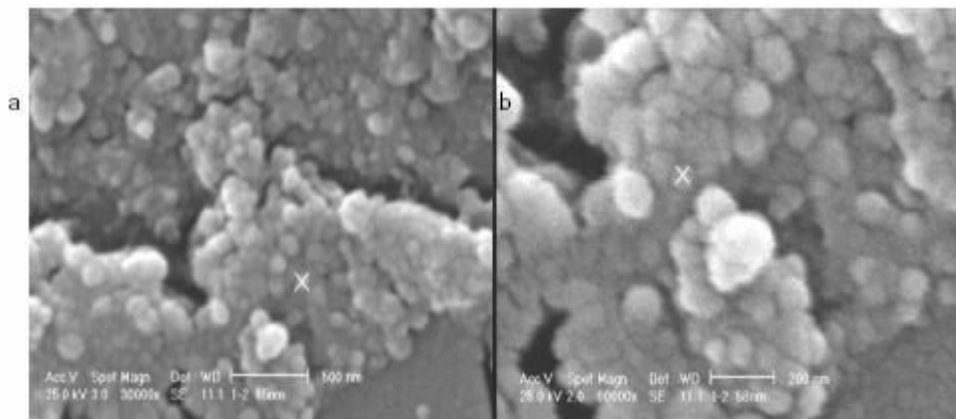


Figure 18: Scanning electronic microscopy of the outer surface of the BSA nanoparticles. a: Outer surface of the particle with magnification of 30000. b: Outer surface of the particle with magnification of 60000.

4.1.1.5. Atomic force Microscopy

Atomic force microscopy is used for observing the surface structure of particles. It used to obtained a resolution in the nanometer range and three-dimensional. Samples undergo relatively

mild preparation procedures that reduce the risk of damaging or altering the sample properties prior to measurement (Burey et al., 2008).

Particles produced using fluid gels typically have irregular shapes and can even have tail like structures (Frith, Garijo, Foster, & Norton, 2002) (Williams et al., 2002). (Burgain et al., 2013) (Fig. 19.20) used AFM to identify specific interactions between bacteria and whey proteins. Force measurements and topography images were made at room temperature and different pH. It was observed that many factors influence “Bacteria / dairy matrix” interactions, including the nature of proteins, the nature of strains and the pH of the media.

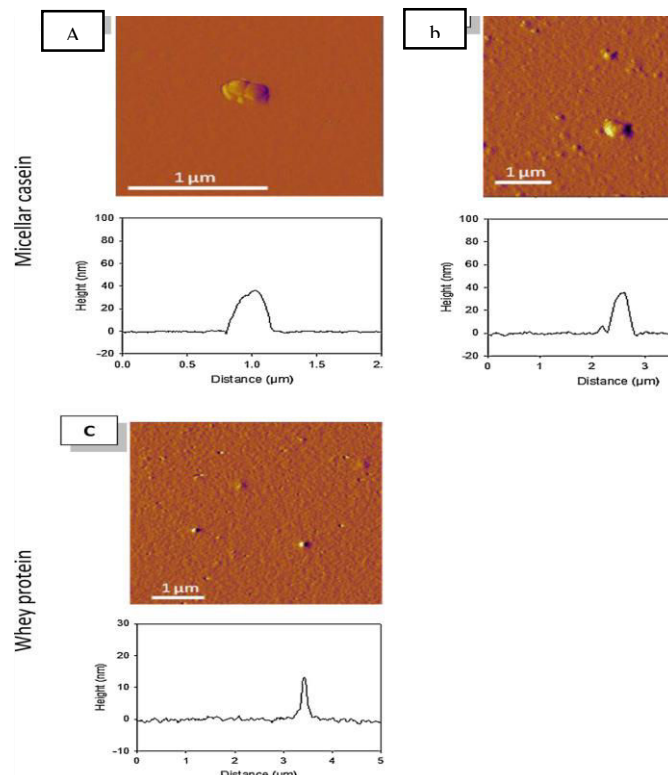


Figure 19: Deflection images of micellar casein at two pHs ((A) pH 6.8 and (B) pH 4.8) and whey proteins ((C) pH 4.8). Each image corresponds to 512 horizontal lines that describe the outward and return of AFM cantilever tip (1024 scans are made on each image). The graphics below each image correspond to height profiles taken from a cross section on the AFM images.

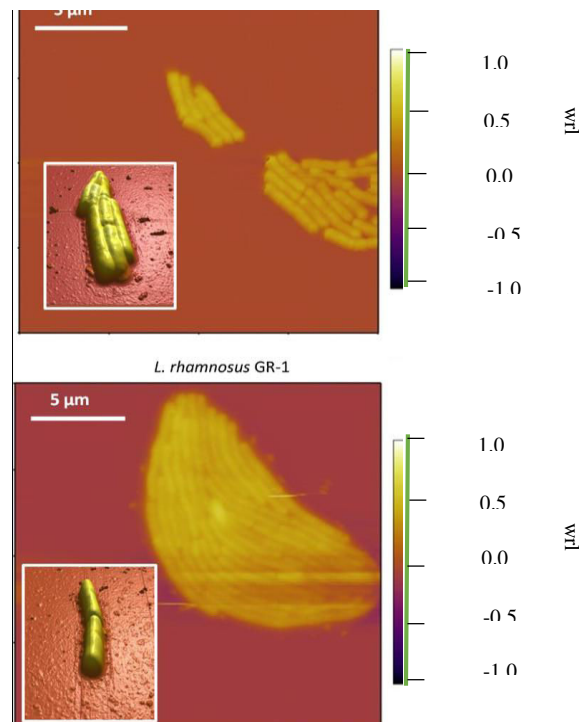


Figure 20: Height images of bacterial strains *L. rhamnosus* GG and *L. rhamnosus* GR-1. Each image corresponds to 512 horizontal lines that describe the outward and return of AFM cantilever tip (1024 scans are made on each image) insets: 3D views of bacterial strains.

4.1.2. Laser light scattering

Laser light scattering also measures the size of microcapsules in the size range between 0.02-2.000 μm.

4.1.2.1. Single particle optical sensing (SPOS)

SPOS is used to measure particle size distribution (Onwulata, 2005); it is based on the magnitude of pulse generated by single particles passing through a small photo zone,

illuminated by light from laser bulb, which can be correlated with the size of the particles (Dodds, 2013).

4.1.2.2. Focused beam reflectance measurement (FBRM)

FBRM provides in situ/online characterization of non-spherical particles by measuring chord lengths of particles (Li, Wilkinson, & Patchigolla, 2005), (Barrett & Glennon, 2002).

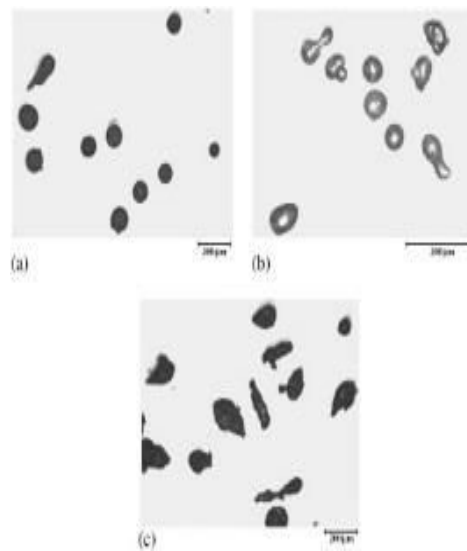


Figure 21: Raw micrographs of samples: (a) ceramic beads; (b) plasma aluminium; and (c) zinc dust.

In Fig 21, FBRM measurements were conducted in aqueous suspensions which were prepared with distilled water. In the image analysis experiments, the particles were evenly distributed on microscope slides and were measured in dry form (Li et al., 2005).

4.1.2.3. Malven Zetasizer

Malven Zetasizer characterized the electrical properties of biopolymer particles by ζ -potential. to evaluate the magnitude of the repulsion between capsules (Legrand, Barratt, Mosqueira, Fessi, & Devissaguet, 1999). and predict the stability of particle suspensions to aggregation

(Jahanshahi & Babaei, 2008) ζ -Potential measurements are highly sensitive to pH and ionic strength. In addition, in systems consisting of a biopolymer mixture, it may be difficult to interpret the data since they will all contribute to the overall signal.

4.2. Microcapsules composition, physical state and release

To determine particle composition and distribution of active ingredients within particles, several techniques have been explored.

4.2.1. FTIR spectroscopy

FTIR gives information on the chemical structures and interactions between the matrix and the active compound (or bacteria). (Ben Messaoud et al., 2015a) used FTIR to investigate molecular interactions between alginate and thickening agents. (Ben messaoud et al., 2015b) studied the influence of the thickening agents for alginate capsules by modulated with anionic chitosan, xanthan gum and maltodextrin. The result showed that the release profile of cochineal red food dye changed considerably with the different thickening agents. After a one-day storage, capsules filled with chitosan avoided any molecular transport and 35% of the encapsulated red dye remained in the capsules filled with maltodextrin.

4.2.2. X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy was used for chemical analysis of particle surface composition, while elemental analysis has been used to study the overall composition of particles and evaluate if a certain compound was encapsulated within the particles. (Montes, Gordillo, Pereyra, & de la Ossa, 2011) used X-ray to determine the particle composition, size

and shape. The powder samples were mounted on double sided adhesive and analyzed without any further treatment.

4.2.3. Differential scanning calorimetry (DSC)

DSC is used to detect the thermal changes in the sample during heating or cooling to show the presence of particular organization as crystals or detect interactions between biopolymers in the particles. (Ribeiro, Silva, Ferreira, & Veiga, 2005) assessed interactions between alginate and chitosan in capsules membranes (Yang et al., 2013).

4.2.4. Rheological gel characterization

The rheometer investigates the influence of polymers composition on gel properties. The elastic (G') and viscous (G'') moduli of gels are investigated by dynamic mechanical analyses with a plate-plate geometry (20 mm) at 20°C. Rheological frequency sweep tests are performed on three-dimension. Dynamic strain sweep tests are measured at a frequency of 1 Hz to investigate the linear viscoelastic range. In general, the elastic modulus of an gel depends on the number of cross-links and length and stiffness of the chains between cross-links (Pongjanyakul & Puttipipatkachorn, 2007) .

4.2.5. Spectrophotometer

Spectrophotometer is used to measure the relative amount of released active compound versus time. At scheduled time intervals, the amount released was determined from solution

absorbance at 500 nm wavelength. At the end of the experiments, to determine the total mass initially encapsulated and the remaining amount, the capsules are destructured by sonication (Leick, Henning, Degen, Suter, & Rehage, 2010).

Table 4: Particle characterization

Particle characterization	Machines	Measurement	References
Particle size	Static light scattering (SLS)	- The intensity of scattered light waves as a function of scattering angle.	
	dynamic light scattering (DLS)	- Size of particles from direction and speed of particle movement due to Brownian motion	
	Confocal Laser Scanning Microscopy (CLSM)	- Provides a better spatial 3D image. - the distribution of polymers and cross-linking ions	(Lamprecht et al., 2000).
	Malvern particle sizing (Mastersizer)	- Particles in size ranging from 0.05 up to 900 μm .	
Particle charge	Single particle optical sensing (SPOS)	- particle size distribution	(Onwulata, 2005)
	Malven Zetasizer	- characterized the electrical properties of biopolymer particles by ζ -potential	(Jahanshahi & Babaei, 2008). (Legrand et al., 1999)
	Conventional optical microscopy	- Characterizing structures of capsules. - Size of capsules.	(Zuidam & Nedovic, 2010b)
Particle morphology	Scanning Electron Microscopy (SEM)	- Surface characteristics, such as composition, shape and size.	(Mohsen Jahanshahi & Babaei, 2008) (Montes, Gordillo, Pereyra, & Martínez de la Ossa, 2011)
	Transmission Electron Microscopy (TEM)	- Structures of very thin samples. - Morphology and shell thickness of encapsulates following fixation, dehydration, and sectioning	(Chen & Subirade, 2005)
	Atomic force microscopy (AFM)	- Surface structure of particles in the nanometer range and three-dimensional.	(Burey et al., 2008)

		(Burgain et al., 2013)	
Particle composition and physical state and release	Confocal Laser Scanning Microscopy (CLSM)	- Provides a better spatial 3D image. - Provides additional information such as the three-dimensional location and quantification of the encapsulated phase.	(Lamprecht et al., 2000).
	Focused beam reflectance measurement (FBRM)	- Provide in situ/on-line characterization of non-spherical particles by measuring chord lengths of particles	(Li et al., 2005a), (Barrett & Glennon, 2002)
	QICPIC	- 2D, 3D view of particles, and then determine several size and shape parameters. - Sphericity, and The convexity the particles	(Cellesi, Weber, Fussenegger, Hubbell, & Tirelli, 2004) (Burgain et al., 2011).
	X-ray photoelectron spectroscopy	- Chemical analysis of particle surface composition, - particle size and shape - presence of compounds in the resulting precipitates.	(Montes, Gordillo, Pereyra, & de la Ossa, 2011)
	Differential scanning calorimetry (DSC)	- Detect crystals of an encapsulated compound, - Interactions between biopolymers in the particles.	(Ribeiro et al., 2005) (Yang et al., 2013)
	Rheological gel characterization	- Rheological parameters of initial preparation - Mechanical parameters of capsules	(Pongjanyakul & Puttipipatkachorn, 2007)
	Spectrophotometer	- The relative amount of released compounds. - Determine the total mass initially encapsulated.	(Leick et al., 2010)

5. Particular interest of LAB encapsulation in Alginate

5.1. Pure alginate capsules

The common materials used in encapsulation of probiotic bacteria involve polysaccharides, originate from seaweed (K-carrageenan, alginate), plants (starch, arabic gum), bacteria (gellan, xanthan), and animal proteins (milk, gelatin).

Alginates are excessively used at laboratory- and industry scale for encapsulation because they are cheap, readily available, biocompatible, and have low toxicity (Krasaekoopt et al., 2003).

There are various techniques to prepare alginate alginate microcapsules, based on dry techniques freeze-drying (Shah & Ravula, 2000, Giulio et al., 2005, Capela, Hay, & Shah, 2006, Ross, Gusils, & Gonzalez, 2008), spray-drying ((K. Y. Lee & Heo, 2000), or electrospraying (Laelorspoen, Wongsasulak, Yoovidhya, & Devahastin, 2014). Humid capsules are also largely used based on liquid form, emulsification and ionic gelation (Hansen, Allan-Wojtas, Jin, & Paulson, 2002, Mandal, Puniya, & Singh, 2006, Allan-Wojtas, Truelstrup Hansen, & Paulson, 2008), extrusion (Ivanova, Chipeva, Ivanova, Dousset, & Poncelet, 2002, Chandramouli, Kailasapathy, Peiris, & Jones, 2004), (Sathyabama, Vijayabharathi, & others, 2014), (Corbo, Bevilacqua, Gallo, Speranza, & Sinigaglia, 2013), (Muthukumarasamy & Holley, 2007).

In comparison with other encapsulation techniques, it was found that the extrusion technique is the best technology for large-scale/industrial applications (Prüsse, Bilancetti, Bučko, Bugarski, Bukowski, Gemeiner, Lewińska, Manojlovic, Massart, Nastruzzi, & others, 2008). It was utilized to prepare spherical microbeads with a hydrophobic core and a hydrophilic or hydrophobic shell (Nicolaas Jan Zuidam & Shimoni, 2010). (Özer, Kirmaci, Şenel, Atamer, & Hayaloğlu, 2009) observed that alginate microencapsulation techniques were effective to protect encapsulated probiotic bacteria. Comparing the number of alive probiotic bacteria free

and encapsulated in cheese, it was observed that the number of encapsulated bacteria decreased approximately 1 log while decreased approximately 3 log with free probiotic bacteria in cheese. (Amine et al. 2014) encapsulated *Bifidobacterium longum* by extrusion/freeze-drying processing and showed that microbeads obtained by freeze-drying were the most effective in preserving bacterial viability with a loss of viability of 2 log after 24 h as compared to extrusion beads and free cells where a loss of 2.9 and 2.75 log were respectively observed. Beads size, alginate concentration, and polymer functionalization can be nevertheless managed to extend probiotic viability.

(Allan-Wojtas, Truelstrup Hansen, & Paulson, 2008) prepared calcium alginate microcapsules, with or without probiotic bacteria using emulsification. Results showed large differences between the alginate matrix of microcapsules without and with bacteria. The presence of bacteria during gelation caused local changes to the gelation process and the occurrence of “void space” phenomenon, as observed in fermented dairy products.

(Chandramouli, Kailasapathy, Peiris, & Jones, 2004) studied the effect of capsule size, sodium alginate concentrations and calcium chloride concentrations on the viability of encapsulated bacteria. The viability of probiotic bacteria in the microcapsules increased with alginate capsule size and gel concentration. No significant differences were observed in the viability of probiotic bacteria in capsules when the concentration of calcium chloride increased.

There are some disadvantages related to the alginate microbeads. For example, the microbeads are very porous which this is a drawback when the aim is to protect the cells from its environment (Gouin, 2004). Moreover, alginate microbeads is sensitive to acidic environment (Mortazavian et al., 2008) which make them not compatible for microparticles in stomach conditions. (Sousa et al., 2015) presented that the alginate micro-particles was not able to exert protection to the encapsulated probiotic cells stored at $-20\text{ }^{\circ}\text{C}$ for 60 days, especially from acid and particularly bile salts. Nevertheless, the defects alginate micro-particles can be treated by

mixing alginates with other polymer compounds, coating the capsules by another compound or applying structural modification by using different additives (Krasaekoopt et al., 2003)

Table 5: Pure alginate capsules

Alginate %	Encapsulation method	Probiotic bacteria	Survival	Stability of capsules	Reference
3%	Freeze dried	<i>Lactobacillus acidophilus</i> MJLA1 and <i>Bifidobacterium</i> spp. BDBB2)	+	+	(Shah & Ravula, 2000).
2, 3, and 4%	Spray drying	<i>Bifidobacterium longum</i> KCTC 3128 and HLC 3742	+	+	Survival of <i>Bifidobacterium longum</i> Immobilized in Calcium Alginate Beads in Simulated Gastric. (K. Y. Lee & Heo, 2000)
10%		<i>Bifidobacterium lactis</i> and <i>Lactobacillus acidophilus</i>	-	-	(Trindade & Grosso, 2000)
1.8% alginate solution	Emulsification and ionic gelification	<i>Bifidobacteria lactis</i> Bb-12, <i>Bifidobacteria adolescentis</i> 15703, <i>Bifidobacteria breve</i> 15700, <i>Bifidobacteria lactis</i> Bb-12 and <i>Bifidobacteria longum</i> Bb-46	+	-	(Hansen, Allan-Wojtas, Jin, & Paulson, 2002)

4%	Extrusion	<i>Enterococcus faecium</i> A 2000	+	+	(Ivanova, Chipeva, Ivanova, Dousset, & Poncelet, 2002)
		<i>Lactobacillus acidophilus</i> DD910 and <i>Bifidobacterium lactis</i> DD920	-	+	(K. Kailasapathy & Sultana, 2003)
0.75 %, 1%, 1.5%, 1.8%, 2%	Extrusion	<i>Lactobacillus acidophilus</i> CSCC 2400 and <i>L. acidophilus</i> CSCC 2409	+	+	(Chandramouli, Kailasapathy, Peiris, & Jones, 2004)
2%	Freeze drying	<i>Lactobacillus acidophilus</i> , <i>Lactobacillus delbrueckii</i> subsp <i>bulgaricus</i> , <i>Streptococcus salivarius</i> subsp. <i>thermophilus</i>	+	+	(Giulio et al., 2005)
2-3-4%	Emulsification	<i>Lactobacillus casei</i> NCDC-298	+	+	(Mandal, Puniya, & Singh, 2006)
2%	Extrusion	<i>Lactobacillus delbrueckii</i> subsp. <i>Delbrueckii</i> ATCC 9646	+	+	(Idris & Suzana, 2006)

	Freeze drying	<i>L. acidophilus</i> , <i>L. casei</i> , <i>L. rhamnosus</i> , <i>B. infantis</i>	+	+	(Capela, Hay, & Shah, 2006)
3%	Extrusion and Emulsion	<i>L. reuteri</i> ATCC 53608	+	+	(Muthukumarasamy & Holley, 2006)
2%	Extrusion	<i>L. acidophilus</i>	+	+	(King, Huang, & Tsen, 2007)
3%	Extrusion	<i>B. Longum</i> , <i>L. reuteri</i>	+	+	(Muthukumarasamy & Holley, 2007)
		<i>B. lactis</i> Bl-04, Bi-07	+	+	(W. K. Ding & Shah, 2007)
		Lactobacillus rhamnosus, Bifidobacterium longum, L. salivarius, L. plantarum, L. acidophilus, L. paracasei,			
2%	Extrusion	<i>Lactobacillus acidophilus</i> LA-5 and <i>Bifidobacterium bifidum</i> BB-12	+	+	(Özer et al., 2008)
2%	Emulsion	<i>Lactobacillus casei</i> (Lc-01) and <i>Bifidobacterium lactis</i> (Bb-12)	+	+	(A. Homayouni, Azizi, Ehsani, Yarmand, & Razavi, 2008)

1.8%	Freeze Drying	<i>LAB</i>	+	+	(Ross, Gusils, & Gonzalez, 2008)
1.8%	Emulsion	<i>Bifidobacterium lactis</i>	+	+	(Allan-Wojtas, Truelstrup Hansen, & Paulson, 2008)
2%	Extrusion	<i>Bifidobacterium bifidum BB-12 and Lactobacillus acidophilus LA-5</i>	+	+	(Özer, Kirmaci, Şenel, Atamer, & Hayaloğlu, 2009)
1%	Emulsion/coating	<i>Lactobacillus acidophilus PTCC1643 and Lactobacillus rhamnosus PTCC1637</i>	+	+	(Mokarram, Mortazavi, Najafi, & Shahidi, 2009)
1.2%	Suspension	<i>Lactobacillus plantarum</i>	+	+	(Bevilacqua, Sinigaglia, & Corbo, 2010)
	Extrusion	<i>Lactobacillus acidophilus ATCC 4356</i>	+		Stability of free and encapsulated <i>Lactobacillus acidophilus</i> ATCC 4356 in yogurt and in an artificial human gastric digestion system. (Ortakci & Sert, 2012)

Alginate-milk 1:1	Extrusion	<i>Lactobacillus bulgaricus</i>	+	+	. (Shi et al., 2013)
2%	Extrusion	<i>Lactobacillus plantarum</i>	+	+	(Corbo et al., 2013)
5g alginate/300 mL distilled water	Freeze-drying	<i>Bifidobacterium longum 15708</i>	+	+	(Amine et al., 2014)
1.4%	electrospraying	<i>Lactobacillus acidophilus</i>	+		(Laelorspoen, Wongsasulak, Yoovidhya, & Devahastin, 2014)
2 g/100 mL sodium alginate	Extrusion	<i>Staphylococcus succinus (MAbB4) and Enterococcus fecium (FIdM3)</i>	+		(Sathyabama et al., 2014)
2%	Extrusion	<i>Lactobacillus casei-01, Lactobacillus paracasei L26, Lactobacillus acidophilus KI and Bifidobacterium animalis BB-12</i>	+	-	(Sousa et al., 2015)

5.2. Capsules produced with alginate mixed with other polymers

The alginate capsules are very porous, and allow diffusion of water in and out of the matrix, so alginate is not fully adapted to protect probiotic bacteria from external environment. Mixing, or coating with other polymers is used to compensate this defect.

(Vodnar & Socaciu, 2014) encapsulated of viable cells in chitosan coated alginate beads. Microencapsulated *L. casei* and *L. plantarum* were resistant to simulated gastric conditions and bile solution. (González-Forte, Bruno, & Martino, 2014) used starch coating alginate to efficiently protect the *L. plantarum* probiotic bacteria through a simulated gastrointestinal system (HCL pH 1–2). (Chan & Zhang, 2005) demonstrated as a novel encapsulation proposal, a coating of alginate micro-particles by hydroxypropyl cellulose. Results showed there was significant improvement in survival of encapsulated cells when exposed to acidic media of pH 1.2 and 2. (Martin, Lara-Villoslada, Ruiz, & Morales, 2013) mixed alginate and starch and showed that the viability of probiotic decreases in 3 log cell numbers in the case of the formulae with alginate only and in 0.3 log in the formulae with mixed alginate and starch. Moreover, the alginate/starch allowed to obtain a suitable particle size and the viability of probiotic was not modified after 45 days at 4 °C. (Rajam, Karthik, Parthasarathi, Joseph, & Anandharamakrishnan, 2012) mixed denatured and undenatured whey proteins with alginate to prepare microencapsules with *Lactobacillus plantarum*. The denatured whey protein encapsulated cells presented a better stability than undenatured whey protein in simulated acidic and bile conditions. This study indicated that combination of denatured whey protein isolate and sodium alginate matrix was able to deliver probiotics with improved survival rate and suitable for controlled core release applications.

(Sandoval-Castilla, Lobato-Calleros, García-Galindo, Alvarez-Ramírez, & Vernon-Carter, 2010) entrapped *Lactobacillus casei* in different beads types made of sodium alginate and

different ratio “amidated low(A)-methoxyl pectin(P)”, by the extrusion technique. The beads made with A–P blends in 1:4 and 1:6 ratios provided a better protection to *Lb. casei* under simulated gastric juice and bile salts. Finally, (Albertini et al., 2010) prepared beads by the extrusion method and nine formulations were developed using alginate as main carrier, xanthan gum (XG) as hydrophilic retardant polymer, and the cellulose derivative, cellulose acetate phthalate (CAP), as gastro-resistant polymer. The results showed that the combination of 0.5% of XG or 1% of CAP within the 3% of alginate solution increased the survival of the probiotic bacteria in acidic conditions from 63% (freeze-dried bacteria) up to 76%.

From all the different results were presented above, it can be concluded that the mixing alginate with other polymers in one matrix or coating alginate capsules with other polymers enhanced the protective properties of the beads, and provided the more stable capsules.

Table 6: Capsules produced with alginate mixed with other polymers

Polymer	Encapsulation method	Probiotic bacteria	Survival	Stability of capsules	Reference
Alginate 3%, skim milk, poly dextrose, soy fiber, yeast extract, chitosan, K-carageenan, and whey (0.6%)	Spray drying	<i>Bifidobacterium longum</i> ATCC 15707, <i>Bifidobacterium infantis</i> ATCC 25962 and <i>Bifidobacterium breve</i> ATCC 15700	+	+	(Yu, Yim, Lee, & Heo, 2001)
Alginate/Skim milk	Freeze drying	<i>Acetobacter xylinum</i>	+	+	(Jagannath, Raju, & Bawa, 2010)
Alginate and Chitosan	Beads coating	<i>Lactococcus lactis ssp. Lactis</i>	+	+	(Klinkenberg, Lystad, Levine, & Dyrset, 2001)
	Extrusion	<i>Lactobacillus rhamnosus</i>	+		(Le-Tien, Millette, Mateescu, & Lacroix, 2004)
	Extrusion	<i>Lactobacillus helveticus</i>	+		(Göksungur, Gündüz, & Harsa, 2005)
	Extrusion/Coating	<i>Lactobacillus acidophilus</i>	+		(K. Kailasapathy & Iyer, 2005)

Extrusion/coating	<i>Lactobacillus acidophilus</i> 547, <i>Bifidobacterium bifidum</i> ATCC 1994, and <i>Lactobacillus casei</i> 01	+	+	(Krasaekoopt, Bhandari, & Deeth, 2006)
Freeze drying/extrusion	<i>Enterococcus faecium</i> MC13	+	+	(Kanmani et al., 2011)
Extrusion/Coating	<i>Bifidobacterium breve</i>	+	+	(Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2011)
Extrusion/coating	<i>Lactobacillus gasseri</i> and <i>Bifidobacterium bifidum</i>	+	+	(Chávarri et al., 2010)
Emulsion/coating	<i>Bifidobacterium breve</i>	+	+	(Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2014)
Extrusion/coating	<i>Lactobacillus casei</i> and <i>Lactobacillus plantarum</i>	+	+	(Vodnar & Socaciu, 2014)
Extrusion/Coating	<i>Lactobacillus plantarum</i> TN8	+	+	(Trabelsi et al., 2013)

	Aerosols/coating	<i>Lactobacillus rhamnosus GG and Lactobacillus acidophilus NCFM</i>	+	+	(Sohail et al., 2011)
alginate beads and the same beads were coated with three types of material, chitosan, sodium alginate, and	Extrusion/Coating	<i>Lactobacillus acidophilus 547, Bifidobacterium bifidum ATCC 1994, and Lactobacillus casei 01</i>	+	+	(Krasaekoopt, Bhandari, & Deeth, 2004)
poly-l-lysine in combination with alginate.					
Sodium alginate or pectin, coated with sodium alginate or chitosan.	Extrusion/coating	<i>Lactobacillus plantarum</i>	+	+	(Brinques & Ayub, 2011)
Alginate/Pectin/chitosan chitosan coated alginate beads	Extrusion/Coating	<i>Lactobacillus plantarum and Bifidobacterium longum</i>	+	+	(Nualkaekul, Lenton, Cook, Khutoryanskiy, & Charalampopoulos, 2012) (Nualkaekul, Cook, Khutoryanskiy, & Charalampopoulos, 2013)

Alginate and pectin	Extrusion	<i>Lactobacillus casei</i>	+	Different percentage	(Sandoval-Castilla, Lobato-Calleros, García-Galindo, Alvarez-Ramírez, & Vernon-Carter, 2010)
	Extrusion/coating	probiotic cells	+	+	(Voo, Ravindra, Tey, & Chan, 2011)
	Freeze dried	<i>Bifidobacterium bifidum</i>	+	+	(Martin-Dejardin et al., 2013)
Alginate and hydroxypropyl cellulose	Freeze drying	<i>L. acidophilus</i>	+	+	(Chan & Zhang, 2002).
	Extrusion/Coating	<i>Lactobacillus acidophilus ATCC 4356</i>	+	+	(Chan & Zhang, 2005)
alginate/starch	Gas-liquid multi-nozzle apparatus.	<i>Lactobacillus rhamnosus</i>	-	-	(Dembczynski & Jankowski, 2002)
	Freeze-dried	<i>L. acidophilus, B. infantis</i>	-		Viability and survival of free and encapsulated probiotic bacteria in Cheddar cheese. (Godward & Kailasapathy, 2003a) Viability and survival of free, encapsulated and co-

		-	-	encapsulated probiotic bacteria in ice cream (Godward & Kailasapathy, 2003b)
				Viability and survival of free, encapsulated and co-encapsulated probiotic bacteria in yoghurt (Godward & Kailasapathy, 2003c)
		+	+	
Extrusion/Coating	<i>Lactobacillus acidophilus</i> and <i>Bifidobacterium lactis</i>	-	+	(K. Kailasapathy & Masondole, 2005)
Emulsion	<i>L. acidophilus</i> , <i>B. lactis</i>	+		(K. Kailasapathy, 2006)
Emulsion/coating	<i>Lactobacillus acidophilus</i> LA1	+	+	(Sabikhi, Babu, Thompkinson, & Kapila, 2010)
Extrusion	<i>Lactobacillus acidophilus</i>	+	+	(Mirzaei, Pourjafar, & Homayouni, 2012)
Extrusion/coating	<i>Lactobacillus casei</i> DSPV 318 T	+	+	(Soto et al., 2011)

	Emulsification/gelation	<i>Lactobacillus fermentum</i> CECT5716	+	+	(Martin, Lara-Villoslada, Ruiz, & Morales, 2013)
	Extrusion/coating	<i>Lactobacillus plantarum</i> CIDCA 83114,	+	+	(González-Forte, Bruno, & Martino, 2014)
	Fluid bed drying	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i> LBC81	+		(Jiménez, Flores-Andrade, Pascual-Pineda, & Beristain, 2015)
	Freeze-dried/coating	<i>Lactobacillus plantarum</i> spp <i>L. plantarum</i> 299v, <i>L. plantarum</i> 800 and <i>L. plantarum</i> CIP A159	+	+	(Gbassi, Vandamme, Ennahar, & Marchioni, 2009)
1% alginate, glycerol + preservatives in MPG membrane	Emulsification	<i>L. casei</i> YIT 9018	+	+	(Song, Cho, & Park, 2003)
Gellan/xanthan gum, Ca-alginate	superposed air stream (air knife technique)	<i>B. lactis</i> , <i>L. acidophilus</i>	+	+	(McMaster & Kokott, 2005)
alginate beads, xanthan–gellan beads	Extrusion	<i>Propionibacterium freudenreichii</i>	+		(Leverrier, Fremont, Rouault, Boyaval, & Jan, 2005)

Alginate and xanthan gum	Spray freeze drying	<i>Lactobacillus acidophilus</i>	+	+	(S.-J. Kim et al., 2008)
Alginate/ xanthan gum	Extrusion/Freeze dried	<i>Lactobacillus acidophilus</i> and <i>Bifidobacterium lactis</i>	+	+	(Albertini et al., 2010)
alginate-inulin-xanthan gum	laminar flow hood	<i>Lactobacillus acidophilus</i>	+	+	(Nazzaro, Fratianni, Coppola, Sada, & Orlando, 2009)
Alginate coated with palm oil and poly-L-lysine	Freeze dryer/coating	<i>Lactobacillus rhamnosus</i> , <i>Bifidobacterium longum</i> , L. <i>salivarius</i> , L. <i>plantarum</i> , L. <i>acidophilus</i> , L. <i>paracasei</i> , B. <i>lactis type Bl-O4</i> , and B. <i>lactis type Bi-07</i>	+	+	(W. k. Ding & Shah, 2009)
alginate; κ-carrageenan	Extrusion	<i>Lactobacillus reuteri</i>	+		(Tsen, Huang, Lin, & King, 2007)
Alginate-whey protein	Freeze drying	<i>Lactobacillus plantarum</i>	+	+	(Gbassi, Vandamme, Yolou, & Marchioni, 2011)

alginate and denatured whey protein	Spray drying Freeze drying	<i>Lactobacillus plantarum mtcc 5422</i>	+		(Rajam, Karthik, Parthasarathi, Joseph, & Anandharamakrishnan, 2012)
Alginate/pectin/whey protein	Alginate beads coating	<i>Bifidobacterium</i>	+	+	(Guérin, Vuilleumard, & Subirade, 2003)

Chapter II: Material and methods

1. Materials

1.1. Microparticles Materials

Sodium alginate from brown algae (viscosity ≤ 0.02 Pa.s for an aqueous solution of 1 % wt at 20 °C) with an M/G ratio of 1.56 and pectin from citrus peel (galacturonic acid ≥ 74 %, Methoxy Groups ≥ 6.7 %) and xanthan gum was purchased from Sigma-Aldrich (France). Calcium chloride dihydrate and sodium chloride were obtained from VWR (International MgbH, Darmstadt, Germany), synthetic medium M17 from Biokar diagnostics (Beauvais, France), D (+) Glucose monohydrate from Merck (Darmstadt, Germany) and glycerol from VWR (AnalaR NORMAPUR, PROLABO, Fontenay-sous-bois, France).

Stock cultures of *Lactococcus lactis* subsp. *lactis* ATCC 11454, a nisin producing-strain, *Micrococcus flavus* DSM 1790 and *Listeria monocytogenes* CIP 82110 were kept frozen (-80 °C) in synthetic media enriched with 30 % glycerol (M17 broth for the LAB and TSB broth (Biokar diagnostics, Beauvais, France) for the others strains).

1.2. Biofilm Materials

Sodium alginate from brown algae (viscosity ≤ 0.02 Pa.s for an aqueous solution of 1 % wt at 20°C), pectin from citrus peel (galacturonic acid ≥ 74 %, Methoxy Groups ≥ 6.7 %) and hydroxypropylmethylcellulose (HPMC) were purchased from Sigma-Aldrich (France). Corn starch was obtained from Roquette Laisa España (Benifaió, Spain) and calcium chloride dehydrate, sodium chloride, glycerol (99.5 % AnalaR NORMAPUR) from WVR International (Darmstadt, Germany). Synthetic medium M17 and PALCAM agar were supplied by Biokar diagnostics (Beauvais, France), D (+) Glucose monohydrate by Merck (Darmstadt, Germany). Stock cultures of *Lactococcus lactis* subsp. *lactis* ATCC 11454 and *Listeria Monocytogenes* CIP 82110 were kept frozen (-80 °C) in synthetic media enriched with 30 % glycerol (M17

Broth for LAB and Tryptone Soy Broth (TSB, Biokar diagnostics, Beauvais, France) for the other strain).

2. Microparticles preparation

2.1. Microbeads (matrix) preparation

Alginate and pectin solutions (1 % (w/w)) were prepared with sterile physiological water (9 % sodium chloride, VWR Belgium) or with sterile M17 broth supplemented with 0.5 % D (+) glucose. Preliminary studies indicate a positive effect of addition of 0.5 % glucose on *L.lactis* growth and nisin production (data not shown).

L.lactis culture was regenerated by transferring a loopful of the stock culture into 10 mL of M17 broth and incubated at 30 °C overnight. A 10 µl aliquot from overnight culture was again transferred into 10 mL of M17 broth and grown at 30 °C to exponential or stationary phase of growth (6 and 48 h respectively). *L.lactis* cells were collected by centrifugation (20 min, 4 °C, 5000 rpm) and diluted to obtain a target inoculum in microbeads of 10^5 CFU.mg⁻¹.

Alginate/pectin hydrogel microspheres were made using the Encapsulator B-395 Pro (BÜCHI Labortechnik, Flawil, Switzerland). In this study five polymers ratios (A/P) were selected: 100/0; 75/25; 50/50; 25/75; 0/100. The encapsulation technology is based on the principle that a laminar flowing liquid jet breaks up into equal sized droplets by a superimposed nozzle vibration. The vibration frequency determined the quantity of droplets produced and was adjusted at 1200 Hz to generate 1200 droplets per second. The flow rate was 3 mL.min⁻¹. A 120 µm diameter nozzle was used for the preparation of beads. Droplets fell in 250 mL of a sterile CaCl₂ solution (100 mM) continuously stirred at 150 rpm to allow microbeads formation. The beads were maintained in the gelling bath for 15 minutes to complete the reticulation process and then were filtered and washed with buffer solution (9 % sodium chloride).

2.2. Microcapsules (core – membrane) preparation

SA pure or with *L.lactis* composed the membrane of the microcapsules. SA (1.3% (w/w)) solution was prepared with sterile physiological water (9% sodium chloride).

L. lactis culture was re-generated by transferring a loopful of the stock culture into 10 mL of M17 broth and incubated at 30 °C overnight. A 10 µL aliquot from overnight culture was again transferred into 10 mL of M17 broth and grown at 30 °C to exponential or stationary phase of growth (6 and 48 h respectively). *L.lactis* cells were collected by centrifugation (20 min, 4°C, 5000 rpm), diluted and added to SA solution to obtain a target inoculum in microspheres of 10^5 CFU.mg⁻¹.

The core of the capsules was composed by XG (0.2% (w/w)) dissolved in sterile physiological water (9% sodium chloride) or sterile M17 broth supplemented with 0.5% D (+) glucose. Preliminary studies indicated a positive effect of addition of 0.5% glucose on *L. lactis* growth and nisin production (data not shown).

Microcapsules were made using the Encapsulator B-395 Pro (BÜCHI Labortechnik, Flawil, Switzerland). The Buchi technology is based on the principle that a laminar flowing liquid jet breaks up into equal sized droplets by a superimposed nozzle vibration. The vibration frequency determined the quantity of droplets produced and was adjusted at 700 Hz to generate 700 droplets per second. A diameter nozzle 200 µm for membrane and 80 µm for core were used for the preparation of capsules. Droplets fell in a CaCl₂ solution (200 mM) to allow microparticles formation. Capsules were maintained in the gelling bath for 15 min to complete the reticulation process and then were filtered and washed with buffer solution (9 % sodium chloride).

3. Physico-chemical characterization of microparticles

3.1. Size

The mean distribution of capsules was measured using a laser light scattering particle size analyzer Mastersizer S (Malvern Instruments Ltd. UK) equipped with a He–Ne laser, a beam of light of 360 nm. The system was able to determine particles in size ranging from 0.05 up to 900 μm . Measurements were achieved in ten replicates for each system. Results were reported as the volume weighted mean globule size $D(4,3)$ in μm :

$$D(4,3) = \frac{\sum n_i d_i^4}{\sum n_i d_i^3} \quad (1)$$

Where n_i was the number of particles; d_i was the diameter of the particle (μm). The $D(4,3)$ was chosen instead of $D(3,2)$ since it is very sensitive to the presence of small amounts of large particles.

3.2. Morphology

Microparticles were observed under an optical microscope (Olympus AX70, Japan) equipped with a camera (Olympus DP70). Dp controller software (version 2.1.1) was used for taking pictures.

Microparticles shape was also determined by using a QICPICTM analyzer (Sympatec MgbH, Clausthal-Zellerfeld, Germany). The analyzer was directly connected to the reactor and made measurements every 5 min during 60 min. The liquid with capsules was pumped into the reactor, passed through the measuring cell, and images were captured and recorded. The results analysis provided 2D, 3D particle, and then determine shape parameters. The diameter of a circle of equal projection area (EQPC) was calculated. It identified the diameter of a circle with

the same area as the 2D image of the particles. As different shaped particles may have the same EQPC, other parameters were used to characterize the particles. The sphericity was defined as the ratio between the EQPC perimeters with the real particle perimeter. The convexity provided information about the harshness of the particle. A particle with smooth edges had a convexity value of 1 whereas a particle with irregular ones had a lower convexity (Burgain et al., 2011). All tests were run in triplicate.

3.3. Mechanical stability

To investigate mechanical stability of alginate microparticles, individual capsules were compressed between two parallel plates. A rotational rheometer Malvern Kinexus pro (Malvern Instruments, Orsay, France) with a plate-and-plate (20 mm) geometry was used. A force gap test was used to compress the microparticles in a droplet of water from 500 to 5 μm with a linear compression speed of 10 $\mu\text{m}\cdot\text{s}^{-1}$. The gap and the normal force being imposed were measured simultaneously at the upper plate. Three replicates were considered for each type of microcapsules.

4. FTIR spectroscopy

Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra of freeze-dried microparticles were acquired using a Tensor 27 mid-FTIR Bruker spectrometer (Bruker, Karlsruhe, Germany) equipped with an ATR accessory (128 scans, 4 cm^{-1} resolution, wavenumber range 4000 - 550 cm^{-1}) and a DTGS detector. The spectral manipulations were performed using OPUS software (Bruker, Karlsruhe, Germany). All tests were run in triplicate.

5. Encapsulation of *L.lactis*

5.1. *L.lactis* survival and nisin activity

L.lactis free and encapsulated in the different microcapsules was placed in physiological water for 10 days at 30°C. Periodically during the storage period bacterial survival and nisin activity inside and outside microparticles were studied.

To analyze bacterial survival and nisin activity inside microparticles, 1 g of capsules was placed in a 0.1 M citrate solution to dissolved hydrogel microspheres by calcium chelation.

Bacterial survival

Serial dilutions were made from microparticles dissolution and physiological water and then poured onto M17 agar. Plates were incubated for 24 hours at 30 °C before colonies were counted. All tests were run six times.

Nisin activity

Micrococcus Flavus DSM 1790 sensitive to nisin was used to evaluate nisin activity. Two successive *M. flavus* cultures in TSBYE medium (TSB: Biomerieux, Marcy l'étoile, France; YE: Biokar diagnostics, Beauvais, France) were made from cryotubes stored at - 80 °C. The optical density (OD) at 660 nm of the culture was measured, then a dilution in TSAYE medium (TSAYE; Bacteriological Agar Type A: Biokar diagnostics, Beauvais, France; Tween 80: Merck Hohenbrunn, Germany) was performed to obtain a final absorbance at 660 nm of 0.01. 12 mL of this medium were poured and plates were placed at 4 °C for 2 h to allow agar solidification. Then, wells were hollowed in the agar using a Durham and 25 µL of liquefied microcapsules and physiological water containing *L.lactis* were deposited in the wells. In parallel, a negative control (M17) was performed. Plates were incubated overnight at 4 °C, and then for 24 h at 37 °C. The inhibition diameters were measured. Results were expressed in cm

and converted into nisin $\text{mg}\cdot\text{mL}^{-1}$ using a standard curve obtained from a commercial solution of nisin (Sigma-Aldrich, St Louis, USA). All tests were run in triplicate.

5.2. Antimicrobial activity of microparticles

Bacterial strain

Culture of *Listeria Monocytogenes* CIP 82110 was regenerated by transferring a loopful of stock culture into 10 mL of TSB and incubated at 37 °C overnight. A 10 μL aliquot from overnight culture was again transferred into 10 mL of TSB and grown at 37 °C to the end of the exponential phase of growth. Subsequently, this appropriately diluted culture was used for the inoculation of the synthetic media containing *L.lactis* free or encapsulated in order to obtain a target inoculum of 10^2 CFU.mL⁻¹.

Antimicrobial activity

A synthetic media, TSBYE broth, was inoculated with *L.monocytogenes* and *L.lactis* free or encapsulated in exponential state in alginate-xanthan capsules enriched with M17 supplemented with 0.5 % glucose. Media was stored at 30 °C for 7 days. *L.monocytogenes* and *L.lactis* counts were examined both immediately after the inoculation and periodically during the storage period. Serial dilutions were made and then poured onto PALCAM agar (Biokar diagnostics, Beauvais, France) and M17 agar plates. Plates were incubated for 48 hours at 37 °C (PALCAM agar) or 24 hours at 30 °C (M17 agar) before colonies were counted. All tests were run in triplicate.

6. Preparation of the bioactive films

The film forming aqueous dispersions (FFD) contained 4 % (w/w) of HPMC or corn starch and glycerol as plasticizer. The hydrocolloid:glycerol mass ratio was 1:0.25 in every case. Polymers were dissolved in distilled water (pH 6.5) under continuous stirring (400 rpm) at 25 °C.

Lactococcus lactis subsp *lactis* ATCC 11454 was used for the preparation of bioactive films. The selection of the strain was based on its antimicrobial activity, its ability to produce nisin, a bacteriocin. Microbial culture was regenerated according methodology described above. Lactic acid bacteria free or encapsulated were incorporated by adding the bacterial cells preparation into the FFD. The ratio was fixed in order to have a final concentration of 3 logs CFU/cm² in dry film. FFD were then placed under magnetic stirring for 5 minutes.

A casting method was used to obtain the polysaccharide films without lactic acid bacteria and bioactive films. FFD were poured onto a framed and levelled PET Petri dishes (85 or 140 mm diameter) and were dried at 25 °C and 40 % relative humidity for approximately 48 hours. Film thickness was controlled by pouring the amount of FFD that will provide a surface density of solids in the dry films of 56 g/m² in all cases. Dry films were peeled off the casting surface and preconditioned in desiccators at 5 °C and 75 % relative humidity (RH) prior to testing. These values of temperature and RH were chosen to simulate the storage conditions of refrigerated coated products.

7. Films characterization

7.1. Moisture content and thickness

After equilibration, films were dried in triplicate at 60 °C for 24 h in a natural convection oven and for 24 h more in a vacuum oven in order to determine their moisture content.

Measurements of film thickness were carried out by using an electronic digital micrometer (0–25 mm, 1 μm).

7.2. Water vapour permeability

Water vapour permeability (WVP) was measured in dry film discs, which were equilibrated at 75 % RH and 5 °C, according to the gravimetric method described in the AFNORNFH00–030 standard (1974). The dry film was sealed in a glass permeation cell containing silica gel, a desiccant. The glass permeation cells were 5.8 cm (i.d.) \times 7.8 cm (o.d.) \times 3.6 cm deep with an exposed area of 26.42 cm². The permeation cells were placed in a controlled temperature (5°C) and RH (75%) chamber via ventilation. The water vapor transport was determined from the weight gain of the cell. After 30 min, steady-state conditions were reached, and weightings were made. To calculate WVTR, the slopes of weight gain as a function of time in the steady state period were determined by linear regression. For each type of film, WVP measurements were replicated three times and WVP was calculated according to Mc Hugh et al. (1993).

7.3. Oxygen permeability

The oxygen permeability of the films (OP) was measured in triplicate by using an oxygen permeation measurement system (Systech Illinois 8100 Oxygen Permeation Analyser, France) at 20 °C and 75% RH (ASTM, 2005). A sample of the film was placed in a test cell and pneumatically clamped in place. Films were exposed to pure nitrogen flow on one side and pure oxygen flow on the other side. An oxygen sensor read permeation through the barrier material and the rate of permeation or oxygen transmission rate was calculated taking into account the

amount of oxygen and the area of the sample. Oxygen permeability was calculated by dividing the oxygen transmission rate by the difference in oxygen partial pressure between the two sides of the film, and multiplying by the average film thickness.

7.4. Mechanical properties

A Lloyd instruments universal testing machine (AMETEK, LRX, U.K.) was used to determine the tensile strength (TS), elastic modulus (EM), and elongation (E) of the films, according to ASTM standard method D882 (2001). EM, TS, and E were determined from the stress-Hencky strain curves, estimated from force-distance data obtained for the different films (2.5 cm wide and 10 cm long). At least six replicates were obtained for each formulation. Equilibrated film specimens were mounted in the film-extending grips of the testing machine and stretched at a deformation rate of 50 mm/min until breaking. The relative humidity of the environment was held constant at 53 % during the tests, which were performed at 25 °C.

7.5. Optical properties

The transparency of the films was determined through the surface reflectance spectra in a spectrophotometer CM-5 (KonicaMinolta Co., Tokyo, Japan). Measurements were taken from three samples in each formulation by using both a white and a black background. The transparency was determined by applying the Kubelka-Munk theory for multiple scattering to the reflection spectra. As each light flux passes through the layer, it is affected by the absorption coefficient (K) and the scattering coefficient (S). Transparency was calculated, as indicated by Hutchings (1999), from the reflectance of the sample layer on a white background of known reflectance and on an ideal black background, through the internal transmittance (T_i).

Colour coordinates of the films, L^* , C_{ab}^* (Equation 1) and h_{ab}^* (Equation 2) from the CIELAB colour space were determined, using D65 illuminant and 10° observer and taking into account R_∞ (Equation 3) which correspond with the reflectance of an infinitely thick layer of the material.

$$C_{ab}^* = \sqrt{a^{*2} + b^{*2}} \quad \text{Equation 1}$$

$$h_{ab}^* = \arctg\left(\frac{b^*}{a^*}\right) \quad \text{Equation 2}$$

$$R_\infty = a - b \quad \text{Equation 3}$$

Finally, the whiteness index (WI) was calculated by applying equation 4.

$$WI = 100 - \sqrt{(100 - L^*) + a^{*2} + b^{*2}} \quad \text{Equation 7}$$

7.6. FTIR analysis

ATR-FTIR spectra of freeze-dried alginate-pectin microbeads without bacteria and preconditioned polysaccharide films were recorded with a Tensor 27 mid-FTIR Bruker spectrometer (Bruker, Karlsruhe, Germany) equipped with an ATR accessory. 128 scans were used for both reference and samples between 4000 and 400 cm^{-1} at 4 cm^{-1} resolution. Spectral manipulations were then achieved using OPUS software (Bruker, Karlsruhe, Germany). Raw absorbance spectra were smoothed using a 13 points smoothing function. After elastic baseline

correction using 200 points, spectra were centred and normalized. All tests were run at least in triplicate.

7.7. Antimicrobial activity of the films against *Listeria monocytogenes*

Bacterial strain

Stock culture of *Listeria monocytogenes* CIP 82110 was regenerated by transferring a loopful into 10 mL of TSB and incubated at 37 °C overnight. A 10 µl aliquot from overnight culture was again transferred into 10 mL of TSB and grown at 37 °C to the end of the exponential phase of growth. Subsequently, this appropriately diluted culture was used for the inoculation of the agar plates in order to obtain a target inoculum of 10² CFU/cm².

Antimicrobial effectiveness of films

The methodology followed for the determination of antimicrobial effectiveness of films was adapted from Kristo, et al. (2008). Aliquots of Tryptone Soy Agar (TSA, Biokar Diagnostics, Beauvais, France) (20 g) were poured into Petri dishes. After the culture medium solidified, properly diluted overnight culture from *L.monocytogenes* was inoculated on the surface and the different films (containing or not *L.lactis*) of the same diameter as the Petri dishes were placed onto the inoculated surfaces. Plates were then covered with parafilm to avoid dehydration and stored at 5 °C for 12 days. *L.monocytogenes* and *L.lactis* counts on TSA plates were examined both immediately after the inoculation and periodically during the storage period.

The agar was removed aseptically from Petri dishes and placed in a sterile plastic bag with 100 mL of tryptone soy water (Biokar Diagnostics, Beauvais, France).The bag was homogenized

for 2 minutes in a Stomacher blender 400 (Interscience, Saint-Nom-La-Breteche, France). Serial dilutions were made and then poured onto M17 agar and PALCAM agar. Plates were incubated for 48 hours at 37 °C before colonies were counted. All tests were run in duplicate.

8. Statistical analysis

A statistical analysis of data was performed through a one-way analysis of variance using Statgraphics® Plus for Windows 5.1. Homogeneous sample groups were obtained by using LSD test (95 % significance level).

Chapter III: Encapsulation of *Lactococcus lactis* subsp. *lactis* on alginate / pectin composite microbeads: effect of matrix composition on bacterial survival and nisin release

Ce chapitre présente l'encapsulation de *Lactococcus lactis* subsp. *lactis* dans des microsphères d'alginate / pectine. Différents ratios d'alginate / pectine ont été utilisés (A / P) ((100/0, 75/25, 50/50, 25/75, 0/100) et des microsphères ont été préparés par extrusion en encapsulant *Lactococcus lactis* subsp. *Lactis* sous 4 conditions : deux états physiologiques différents (phase exponentielle, phase stationnaire) et en présence de milieux de croissance différents (glucose enrichi M17, l'eau physiologique).

L'objectif de cette étude était d'élaborer les microsphères alginate / pectine et d'évaluer le meilleur ratio de polymères permettant la survie microbienne, la libération de nisine, l'activité anti-listéria en comparaison entre *L.lactis* libre et *L.lactis* encapsulée.

Des solutions d'alginate et de pectine (1% (p / p)) ont été préparées avec de l'eau physiologique (chlorure de sodium à 9%) ou avec un bouillon stérile M17 additionné de 0,5% de D (+) glucose. 10^5 CFU.mg⁻¹ de *L. lactis* sont inoculés dans les microsphères.

La caractérisation physico-chimique des microsphères a consisté à suivre le diamètre, la sphéricité et la convexité des billes de 0 à 7 jours à 30°C. Toutes les microsphères ont été produites dans les mêmes conditions, mais la taille des perles a été montrée dépendante de la viscosité du fluide injecté. Au bout de 7 jours, les billes A/P : 100/0, 75/25, 50/50 ont été observées plus stables que d'autres billes.

La survie et la croissance de *L.lactis* dans les microsphères pendant 7 jours à 30°C ont été mesurées. La survie de *L.lactis* a été évaluée suivant trois facteurs : l'état physiologique de *L. lactis* au moment de l'encapsulation, la composition interne en microsphères (eau physiologique ou M17 enrichi avec 0,5% de glucose), le rapport A / P. La population de *L. lactis* augmente plus rapidement avec le milieu M17 enrichi en glucose par rapport à l'eau physiologique utilisée pour la dissolution des polymères. Le ratio de polymères et l'état physiologique de la bactérie ne semblent pas avoir d'influence significative.

Il a été observé que des billes d'alginate/pectine fournissent une meilleure protection de *L.lactis* contre les facteurs environnementaux que celles faites avec de l'alginate pure ou de la pectine pure. Le ratio A/P 75/25 présente de meilleurs résultats de maintien d'une population stable au sein des perles, en raison de propriétés mécaniques plus stables des microsphères.

La production et l'activité de la nisine a été déterminée pour les différents rapports A/P dans l'eau physiologique à 30°C pendant 7 jours. Les résultats montrent que plusieurs facteurs agissent significativement, comme l'état physiologique et la composition de la matrice en polymères et en substances nutritive). Les meilleurs résultats ont été montrés lorsque *L.lactis* a été encapsulée en phase exponentielle avec A/P (75/25) en présence du M17 enrichi en glucose. Cette activité a été mesurée via l'inhibition de la croissance de *L. monocytogenes* pendant toute la période de stockage. La réduction de la population de *L.monocytogenes* était plus élevée avec des bactéries encapsulées que les cellules libres.

En conclusion, les meilleurs résultats ont été globalement obtenus avec des microsphères (ratio:75/25) enrichies en M17 et complété avec 0,5% de glucose contenant *L. lactis* en phase exponentielle.

Abstract

Alginate/pectin hydrogel microspheres were prepared by extrusion based on a vibrating technology to encapsulate bacteriocin-producing lactic acid bacteria. Effects of both alginate/pectin (A/P) biopolymers ratio and physiological state of *Lactococcus lactis* subsp. *lactis* (exponential phase, stationary phase) were examined for nisin release properties, *L. lactis* survival and beads physico-chemical properties. Results showed that A/P composites were more efficient to increase beads properties than those formulated with pure alginate or pectin. Association of alginate and pectin induces synergistic effect which improves microbeads mechanical properties. FTIR spectroscopy confirms possible interactions between alginate and pectin during inter-penetrating network formation. Physiological state of bacteria during encapsulation process and microbeads composition (A/P ratio, enrichment of internal medium with nutrients) were determining factors for both bacteria viability and bacteriocin release. Of the several matrices tested A/P (75/25) with glucose-enriched M17 gave the best results when *L. lactis* was encapsulated in exponential state.

Keywords: hydrogel microspheres, biopolymers, lactic acid bacteria, physico-chemical properties, antilisterial activity.

1. Introduction

The interest in application of lactic acid bacteria (LAB) in the prevention of food spoilage and foodborne pathogens growth has increased in the last twenty years (Scannell et al., 2000). Many studies have shown that LAB can reduce the presence of *Listeria monocytogenes* in meat and seafood (Budde et al., 2003; Jacobsen et al., 2003; Tahiri et al., 2009) or inhibit other foodborne pathogens such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella Typhimurium*, *Salmonella Enteritidis* and *Staphylococcus aureus* (Trias et al., 2008). Several mechanisms, such as lactic acid production, competition for nutrients or production of antimicrobial compounds, explain inhibition of spoilage or pathogenic microorganisms by LAB. Among LAB, *L. lactis* subsp. *lactis* is particularly used for food preservation because of its ability to produce bacteriocin, such as nisin, to control spoilage and pathogenic bacteria. However the possible interactions between food components and LAB decreased their effectiveness. The immobilization of LAB by encapsulation using natural polymers as proteins or polysaccharides appears as an interesting strategy to protect strain and modulate nisin release.

Encapsulation of bacteria in calcium alginate beads is one of the most studied system for probiotic immobilization and protection (Léonard et al., 2014; Madziva et al., 2005; Polk et al., 1994; Smrdel et al., 2008). Some studies focus on the interest to design composite systems by associating several biopolymers as pectin and alginate to control active components release (Jaya et al., 2008). Authors reported that an increase in pectin caused the diminution of gel barrier and increases the percentage of drug release. Moreover, morphology of alginate pectin microcapsules showed porous micro- structure and they also facilitates active components release.

Sodium alginate is a water soluble anionic polysaccharide, mainly found in the cell walls of brown algae and can be isolated from the bacteria *Pseudomonas* (Pawar and Edgar, 2012). This natural polymer possesses several attractive properties such as good biocompatibility, wide availability, low cost, and simple gelling procedure under mild conditions. Alginate composition is variable and consists in homopolymeric blocks alternating 1,4-linked β -D-mannuronic acid (M) and α -L guluronic acid (G) residues. Physical properties of alginate are dependent on the composition, sequence and molecular weight. Gel formation is driven by interactions between G-blocks which associate to form firmly held junctions due to divalent cations. In addition to G-blocks, MG blocks also participate by forming weaker junctions.

Pectin is one of the main structural water-soluble polysaccharides of plant cell walls. It is commonly used in the food industry as gelling and stabilizing agents. Basically, pectins are polymers of (1- 4) linked partially methyl esterified α -D-galacturonic acid (Synytsya et al., 2003). Pectins gelation is driven by the interaction between the polygalacturonate chains and divalent cations and is described by the egg-box model where the divalent cations are thought to be held in the interstices of adjacent helical polysaccharide chains (Braccini and Pérez, 2001).

Therefore, the objectives of the present study were (a) to develop novel alginate-pectin hydrogel microspheres for the microencapsulation of *L. lactis* subsp. *lactis*, a lactic acid bacteria, by dripping using the vibrating technology (b) to analyze physicochemical properties of composite microbeads (c) to evaluate the effect of polymers ratio and physiological state of encapsulated bacteria (exponential or stationary phase) on microbial survival, nisin release and antilisterial activity (d) to determine if a nutritional enrichment of hydrogel matrix by addition of synthetic media (M17) supplemented with 0.5 % glucose can improve results.

2. Results and Discussion

2.1. Physico-chemical characterization of microbeads

2.1.1. Shape and size

Microscopic images of A/P composite microbeads were presented on Fig.22. Microbeads were fairly regular and spherical. Beads diameter, sphericity and convexity at 0 day and after 7 days at 30 °C were reported on Table 7. In general, the size and shape of microspheres depended on the intrinsic properties of the injected polymer solutions such as viscosity, density and surface tension (Chan et al., 2009).

Initially, little differences were observed in terms of microbeads size and sphericity, but convexity increased clearly with pectin content in the matrix.

After 7 days, significant differences were observed in size and convexity. Convexity provides information about the roughness of the particle. Convexity values are between 0 and 1. A particle with smooth edges has a convexity value of one whereas a particle with irregular ones has a lower convexity (Burgain et al., 2011). After 7 days at 30 °C, sphericity also decreased with pectin content. The sphericity value “1” is a perfect sphere and a particle with a sphericity close to “0” is highly irregular. Therefore, sphericity is a good way to describe particle shape deviation. Alginate contributed largely to beads sphericity as observed by Sandoval-Castilla et al. (2010). As observed in the present paper, Díaz-Rojas et al. (2004) reported that the use of both polymers pectin/alginate in matrix composite beads induce loss of sphericity as the proportion of pectin in the matrix increased, as a consequence of the weaker mechanical stability of the calcium-pectinate network compared to that from calcium-alginate.

Particularly significant changes occurred for pure pectin microbeads. A swelling phenomenon was responsible for the changes in size as no aggregation occurred. 100 % of pectin microbeads were distorted and became more irregular.

From results in Table 7, it was concluded that the beads A/P 100/0, 75/25, 50/50 were more stable than other beads with higher pectin content.

Table 7: Size, sphericity and convexity of microbeads with different ratios of polymers, at day 0 and after a storage period in physiological water of 7 days at 30 °C.

A/P	Size D[4,3] (µm)		Sphericity		Convexity	
	day 0	day 7	day 0	day 7	day 0	day 7
100/0	264 (13) ^{ax}	255 (5) ^{ax}	0.90 (0.01) ^{ax}	0.93 (0.01) ^{ax}	0.25 (0.01) ^{ax}	0.23 (0.04) ^{ax}
75/25	274 (10) ^{ax}	267 (3) ^{bx}	0.90 (0.02) ^{ax}	0.90 (0.01) ^{ax}	0.27 (0.01) ^{ax}	0.24 (0.05) ^{ax}
50/50	275 (2) ^{ax}	268 (7) ^{bx}	0.95 (0.01) ^{bx}	0.90 (0.02) ^{ay}	0.43 (0.01) ^{bx}	0.35 (0.06) ^{bx}
25/75	272 (11) ^{ax}	285 (6) ^{cx}	0.94 (0.01) ^{bx}	0.88 (0.01) ^{ay}	0.50 (0.01) ^{cx}	0.50 (0.01) ^{cx}
0/100	269 (15) ^{ax}	299 (10) ^{cy}	0.93 (0.02) ^{abx}	0.75 (0.01) ^{by}	0.59 (0.02) ^{dx}	0.70 (0.01) ^{dy}

a, b, c, d Different letters indicate significant differences between the various samples among the same column (p <0.05)

^{x,y,z} Different letters in the same file indicate significant differences among time for a same sample (p <0.05)

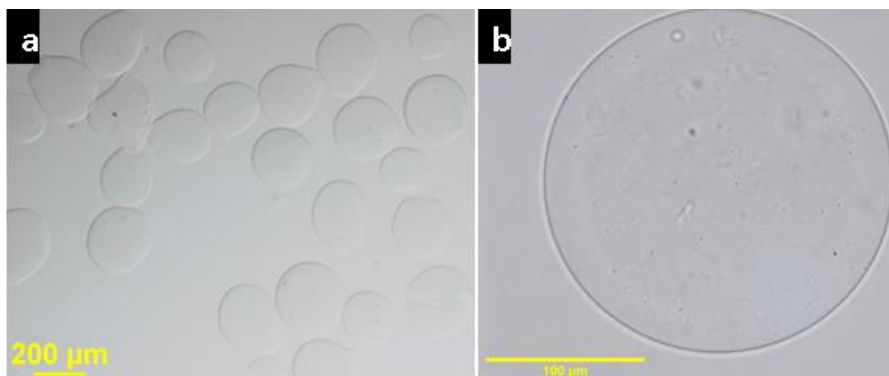


Figure 22: Microscopic images of A/P (75/25) beads population (a) and a single microbead (b).

2.1.2. Mechanical stability

The microcapsules functionality is closely related to their chemical and mechanical stability. In fact microspheres are sensitive to deformations that may lead to their rupture or to undesirable early release of their contents. In order to evaluate the mechanical stability of the prepared systems, microbeads were compressed between two parallel plates. As shown in Fig.2, the normal force (N) versus the gap distance (mm) is plotted as double-logarithmic compression curves (Degen et al., 2011).

As expected, for all the systems, the normal force increases with a decreasing gap, because the bead became more and more compressed. However, some differences in the force evolution were observed. In fact the initial force at 0.2 mm could be noted for the different systems which could be related to the beads size.

Some curves showed a region of force diminution at small gaps (<0.005 mm) which could be related to potential beads rupture. Nevertheless, due to the low speed used in these experiments, microbeads break-up was not clearly observed.

For comparison purpose, at an intermediate gap (0.01) the beads 25/75 and 75/25 showed a better stability than the others systems were a force of 0.5 N is needed to main the beads at the corresponding gap in Fig.23.

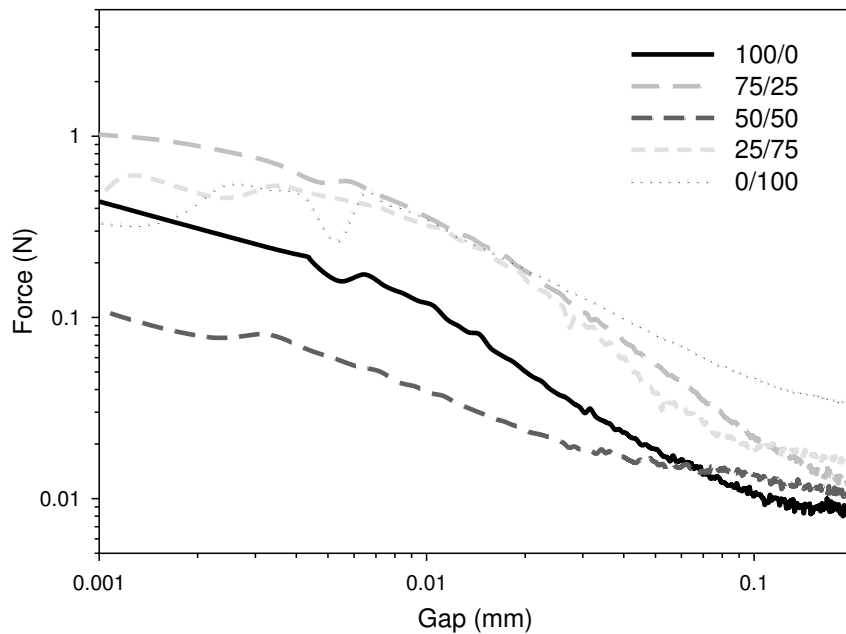


Figure 23: Force/gap curves of alginate, pectin and alginate/pectin microbeads

The differences on mechanical stability could be related to potential synergy between alginate and pectin (Walkenström et al., 2003). This synergism is attributed to a heterogeneous association of the G blocks of alginate and the methyl ester regions of pectin (Oakenfull et al., 1990). In the other hand, molecular modeling showed that the G blocks and methylesterified polygalacturonic acid ribbons could pack together in parallel twofold crystalline arrays (Thom et al., 1982).

Practically, all the force/gap curves showed a region where the force decreased at small gaps (<0.005 mm) which could be related to potential beads rupture at a compressed state. For alginate beads, the start of hydrogel break-down is associated with the rupture of one single polymer chain (Zhang et al., 2007).

It's interesting to note, that the bursting zone was less obvious for composite alginate/pectin beads which are stabilized by alginate G, MG junctions and pectin polygalcturonic junctions. The formation of IPN (Inter-Penetrating Network) could lead to stiffer microbeads.

2.1.3. FTIR spectroscopy

Fig. 24a shows FTIR spectra of freeze-dried alginate, pectin and alginate/pectin mixture and Fig. 24b spectra of the corresponding freeze-dried microbeads. As shown in Fig.24a, alginate spectra (100/0) displayed two vibrations in the infrared spectrum due to the carboxylate group; an antisymmetric stretch at 1600 cm^{-1} and a symmetric stretch at 1412 cm^{-1} (Sartori et al., 1997). The pectin spectra (0/100) displayed also a peak between 1720 and 1760 cm^{-1} . The region between 1000 and 1140 cm^{-1} corresponds to the stretching vibrations of (C–OH) side groups and the (C–O–C) glycosidic bond vibration (Kamnev et al., 1998). The absorption bands between 1100 and 1200 cm^{-1} were from ether (R–O–R) and cyclic C–C bonds in the ring structure of pectin molecules. The regions between 1590 and 1600 cm^{-1} are due to the aromatic ring stretching. The region between 1600 and 1800 cm^{-1} is of special interest and was usually used to compare pectin samples (Synytsya et al., 2003). This spectral region reveals the existence of two bands at 1620 – 1650 and 1720 – 1760 cm^{-1} from free and esterified carboxyl groups, respectively.

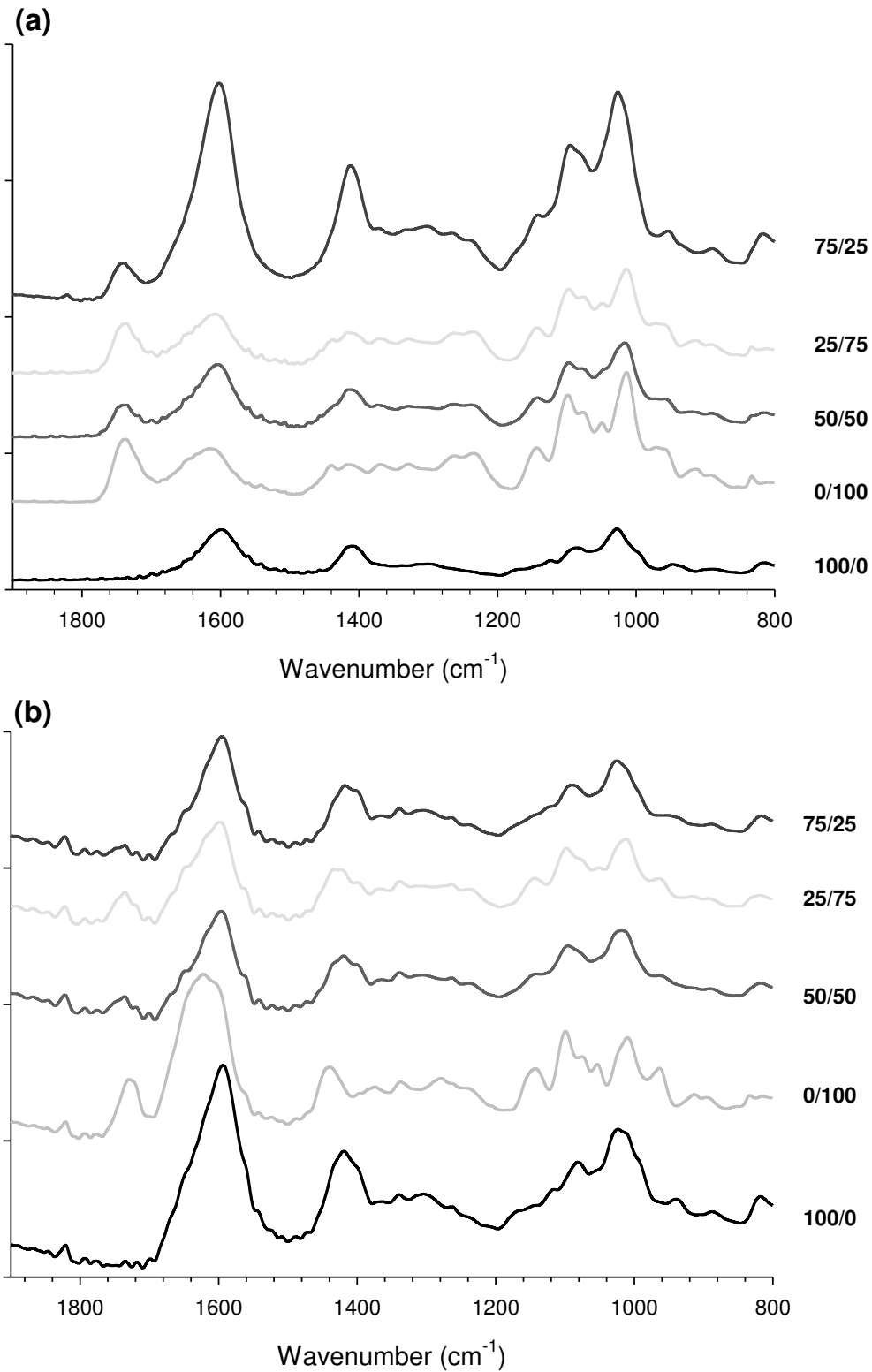


Figure 24: FTIR spectra of alginate, pectin and alginate/pectin solutions (a) and alginate, pectin and alginate/pectin composite microbeads (b).

For full assignment of the infrared bands of alginate and pectin is presented in Table 8. As expected, the mixture solutions of alginate/pectin (75/25, 50/50 and 25/75) displayed the typical bands of the two corresponding biopolymers without significant shifts. However some differences in band intensities were noticed. In particular the mixture 75/25 showed a significant increase of the bands corresponding to the carboxylate groups of pectin and alginate (1600 and 1400 cm^{-1}) and to the glycosidic pectin vibrations ($1000\text{-}1300\text{ cm}^{-1}$).

The IR spectra of calcium alginate beads (100/0) (Fig.24b) displayed the same characteristic peaks of sodium alginate with a greater intensities of the bands corresponding to the carboxylate groups (1600 and 1412 cm^{-1}) (Pereira et al., 2003). The infrared spectrum of calcium pectinate microbeads (0/100) showed intensity increase of the band corresponding to the carboxylate groups (1620 cm^{-1}) and the appearance of a narrow peak at 1400 cm^{-1} due to the interactions between the galacturonic residues and divalent cations (Braccini and Pérez, 2001). The alginate/pectin blend beads, showed the same typical bands of alginate and pectin with some differences as function of the alginate/pectin ratio especially in the wavenumber between 1000 and 1200 cm^{-1} . In fact, FTIR analysis of 75/25 beads showed the same two bands (1020 and 1060 cm^{-1}) as alginate 100/0. The increase of pectin amount resulted in the appearance of a shouldering peak at 1145 cm^{-1} for (50/50) and then in well-defined peak for the 25/75 system.

Moreover the pH-values of the studied mixtures 100/0, 0/100, 75/25, 50/50 and 25/75 were 7.04, 3.30, 4.34, 3.94, 3.60 for, respectively. Alginate and pectin could form synergistic mixed gels at low pH values (but >4) in the absence of calcium with a relatively low gelation kinetic (>200 min) (Walkenström et al., 2003). In our case the alginate/pectin mixture was not allowed to stand, and the formation of composite gel was not observed, however we could suppose the possible formation of cooperative bands between alginate and pectin for mixtures before the encapsulation step, which could explain the increase of the intensity of the characteristics bands

and therefore better physico-chemical properties. Otherwise, the freeze-drying step of the hydrogel microspheres before the FTIR study could also initiate or reinforce the potential interactions between the two polymers.

Table 8: FTIR Bands of alginate and pectin with Assignments.

Polymer	Wavenumber (cm ⁻¹)	Functional groups
Alginate	1600	COO ⁻ antisymmetric stretch
	1412	COO ⁻ symmetric stretch
	1024	COC antisymmetric stretch
Pectin	1740	C=O stretching
	1640-1610	COO ⁻ antisymmetric stretching
	1440	COO ⁻ symmetric stretch
	1380	C-H bending
	1240	CO, CC of ring stretching
	1145	COC of glycosidic link/ring
	1100	CO, CC, CCH, OCH ring

2.2. Encapsulation of *L.lactis*

2.2.1. *L.Lactis* survival

The suitability of different matrices for *L.lactis* survival and growth inside and outside hydrogel microspheres was studied in physiological water for 7 days at 30 °C (Fig. 25 and 26). Bacterial viability significantly changes with three factors: A/P ratio, physiological state of *L.lactis* during the bead formation and internal composition of the microsphere (physiological water or M17 enriched with 0.5 % glucose). Non-encapsulated *L.lactis* was used as control (Fig. 27). Bacterial population decreased significantly for the storage period at 30 °C independently of the physiological state of the strain at the beginning of the assay. The addition of nutrients within microbeads led to significant differences in terms of *L.lactis* counts inside and outside beads. As expected, the population decreased more rapidly with physiological water than with glucose-enriched M17 as medium used for polymers dissolution, independently of polymers ratio and *L.lactis* physiological state.

Encapsulation of *Lactococcus lactis* subsp. *lactis* on alginate / pectin composite microbeads

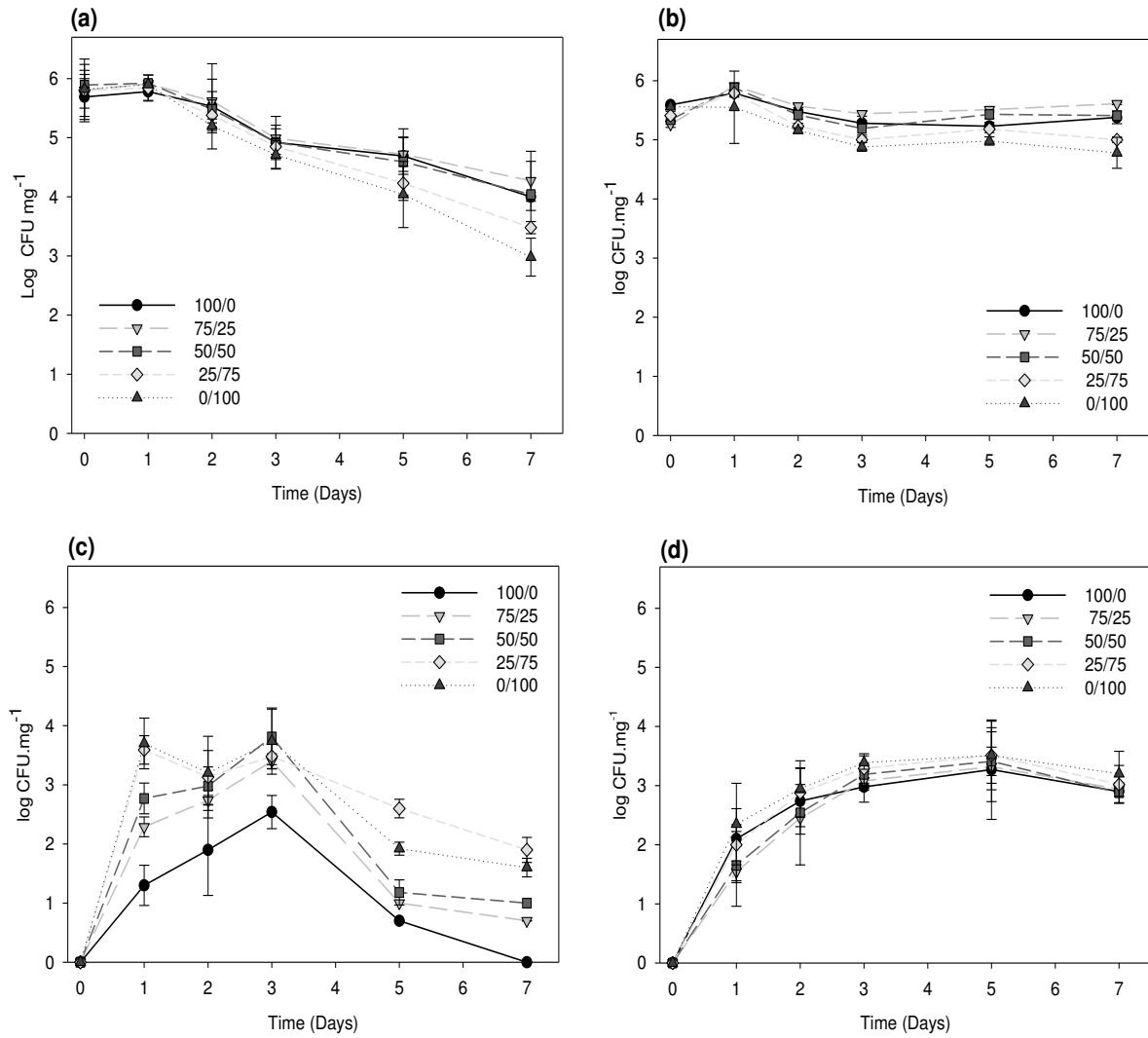


Figure 25: Survival of *Lactococcus lactis*, encapsulated in exponential state, during a storage period in physiological water of 7 days at 30 °C (▲ 100/0, X 75/25, □ 50/50, ★ 25/75, ● 0/100)

- (a) *L.lactis* inside physiological water microbeads (b) *L.lactis* inside glucose-enriched M17 microbeads
(c) *L.lactis* outside physiological water microbeads (d) *L.lactis* outside glucose-enriched M17 microbeads.
Mean values and standard deviations.

From the 5th day of storage bacterial population decreases dramatically inside microbeads, a reduction of approximately 50 % was observed when strain was encapsulated in exponential phase. This decrease was less marked with strain in stationary phase. Concerning bacterial physiological state at the moment of encapsulation, stationary and exponential phase, *L.lactis* counts inside microbeads after 7 days of storage was higher when LAB were encapsulated in exponential phase than in stationary phase. Results focused on the importance of two parameters, physiological state and presence of nutrients. These factors impact certainly cellular stress and therefore bacterial survival.

Finally, the composition of the matrix (A/P ratio) modified significantly bacterial population inside and outside hydrogel microspheres. The use of pectin leads to significant variations especially when physiological water was used for polymers dissolution. Composite A/P hydrogel microspheres tend to present interesting properties compared to pure alginate or pectin beads. Sandoval-Castilla et al. (2010) observed that calcium-alginate-pectin beads matrices provided a better protection against adverse environmental factors to *Lactobacillus casei* than those made with pure alginate or pectin. The lack of alginate in beads reduced the protective effect, suggesting that both polymers, alginate and pectin, form a structured trapping matrix that is more resistant especially to acids.

In this study, microbeads 75/25 present best results to maintain microbial counts within beads. This is could be related to mechanical properties (Fig.1) or to potential reduction of pore size of the composite system which will increase the retention of the encapsulated bacteria. The best mechanical properties were found for A/P 75/25, beads were more stable. With this matrix composition LAB are certainly better retained in microbeads. Outside hydrogel microspheres, *L. lactis* counts change significantly with polymers ratio: bacterial population was higher for microbeads 0/100 and 25/75. Alginate has more linear and organized chains than pectin,

reticulation links were more efficient with calcium ions. This higher crosslinking for alginate increased the cohesive forces between chains (Silva et al., 2009) and difficult bacteria release. In addition, a greater swelling can occur with pectin, also increasing the output of LAB. Previous studies reported a greater swelling of pectin films, compared to alginate films, as a lowering in crosslinking extent allowed more water absorption (Silva et al., 2009; Sriamornsak and Kennedy, 2008).

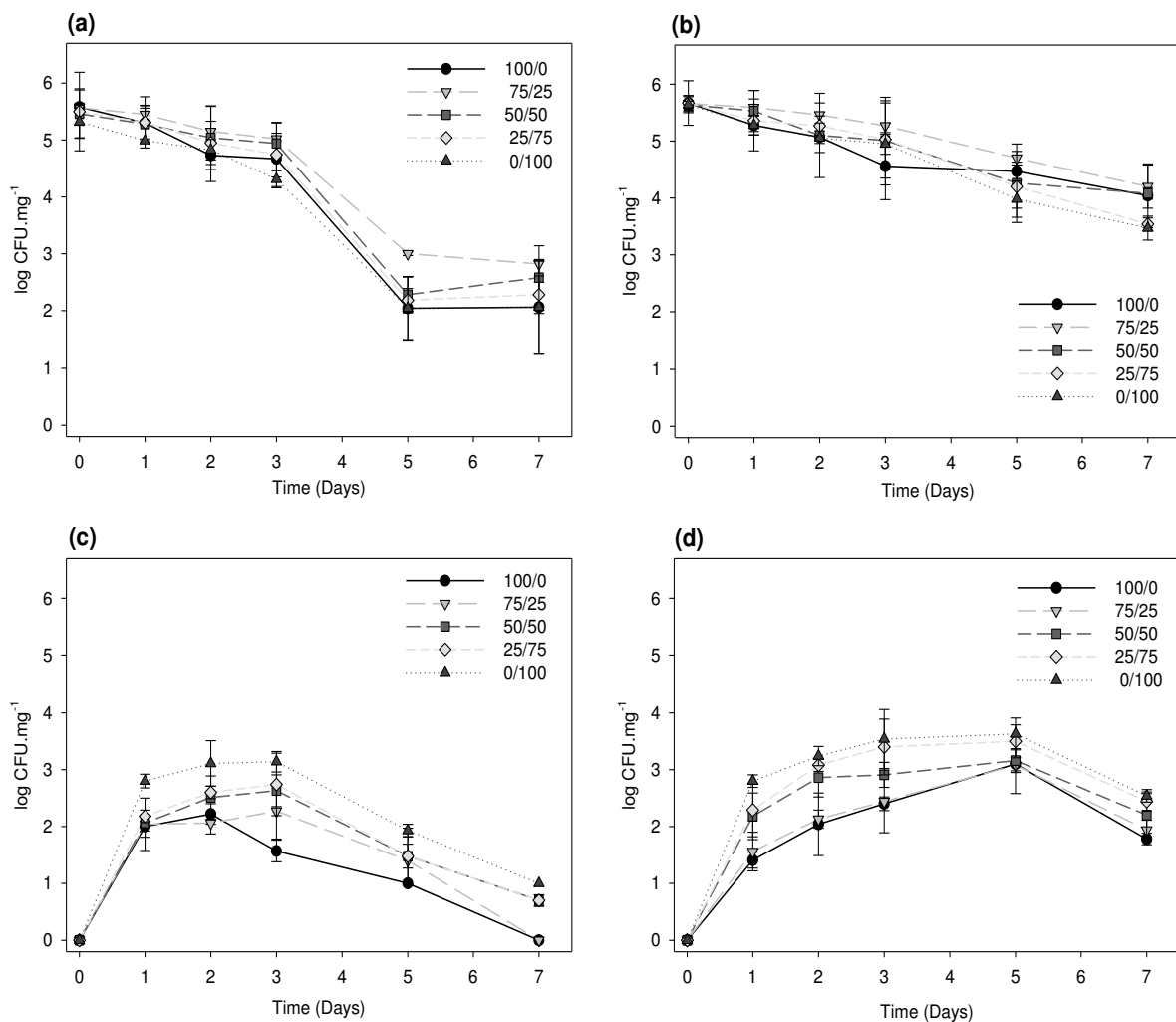


Figure 26: Survival of *Lactococcus lactis*, encapsulated in stationary state, during a storage period in physiological water of 7 days at 30 °C (▲ 100/0, x 75/25, □ 50/50, ★ 25/75, ● 0/100)

(a) *L.lactis* inside physiological water microbeads (b) *L.lactis* inside glucose-enriched M17 microbeads (c) *L.lactis* outside physiological water microbeads (d) *L.lactis* outside glucose-enriched M17 microbeads. Mean values and standard deviations.

2.2.2. Nisin activity

Nisin activity inside and outside microbeads was determined for the different A/P ratios in physiological water at 30 °C for 7 days (Tables 9 and 10).

As observed for bacterial survival, strain physiological state as well as matrix composition (polymers ratios and addition of nutrients) are key factors.

When bacteria were encapsulated in stationary state, a concentration of active nisin was detected inside microbeads at day 0 because nisin was produced before encapsulation step. However when *L.lactis* was encapsulated during the exponential phase of the growth curve, no nisin was detected initially in beads but after 1 day active nisin was present. Nisin production occurs during the bacterial growth and the amount of peptide adsorbed on cell surface is higher during stationary phase compared to exponential phase of bacterial growth curve. Therefore the physiological state of the strain at the time of encapsulation impacts the initial concentration of active nisin in microbeads.

During storage period, no nisin was detected after 3 days of storage inside microspheres prepared with physiological water whatever their polymer composition. The enrichment of microbeads internal media with glucose-enriched M17 improves these data. After a storage period of 7 days a significant amount of antimicrobial peptide was detected independently of physiological state of the bacteria and polymers ratio used. Previous studies reported equally changes in term of bacteriocin concentration with the composition of the nutrient broth (Parente and Ricciardi, 1999).

The A/P ratio does not significantly affect the concentration of active nisin inside microspheres. However, microbeads release properties were modified by the ratio A/P. The mixed matrix (alginate-pectin) reveals an improved suitability to nisin production than pure alginate or pectin microbeads, due to intermediate properties of diffusion and stability of the bead wall. After a

Encapsulation of *Lactococcus lactis* subsp. *lactis* on alginate / pectin composite microbeads

storage period of 3 days at 30 °C two ratios seemed interesting independently of others factors (physiological state of the strain and addition of nutrients): 50/50 and 25/75.

In conclusion, the physiological state of the bacteria during the encapsulation process and the composition of the microbeads (A/P ratio, enrichment of internal medium with nutrients) were determining factors for both bacteria viability and bacteriocin activity which could be related with the nutritional or cellular stress-producing effects. Of the several matrices tested A/P (75/25) with glucose-enriched M17 gave the best results when *L.lactis* was encapsulated in exponential state.

Table 9: Concentrations of active nisin for 3 days at 30 °C inside and outside hydrogel microspheres containing *L.lactis* encapsulated in exponential state. Two internal compositions of microbeads were tested: physiological water and M17 enriched with 0.5 % glucose

A/P	internal microbeads composition	active nisin (mg.mL ⁻¹)							
		day 0	inside microbeads			outside microbeads			
			day 1	day 3	day 7	day 0	day 1	day 3	day 7
100/0	physiological water	–	1.1 (0.3) ^a	–	–	–	–	–	–
75/25		–	1.4 (0.2) ^a	–	–	–	–	–	–
50/50		–	1.2 (0.2) ^a	–	–	–	–	–	–
25/75		–	1.0 (0.2) ^a	–	–	–	–	–	–
0/100		–	0.1 (0.1) ^b	–	–	–	–	–	–
100/0	M17 enriched with 0.5 % glucose	–	0.5 (0.2) ^{ax}	0.9 (0.5) ^{ax}	0.7 (0.4) ^{ax}	–	0.9 (0.2) ^{ax}	0.5 (0.2) ^{ax}	–
75/25		–	0.8 (0.5) ^{ax}	2.2 (0.3) ^{by}	2.2 (0.3) ^{by}	–	2.1 (0.4) ^{bx}	1.7 (0.3) ^{by}	–
50/50		–	0.9 (0.4) ^{ax}	1.8 (0.2) ^{by}	2.0 (0.3) ^{by}	–	1.9 (0.3) ^{bx}	1.9 (0.2) ^{by}	–
25/75		–	1.1 (0.13) ^{ax}	1.8 (0.4) ^{by}	2.0 (0.2) ^{by}	–	1.8 (0.4) ^{bx}	1.9 (0.1) ^{by}	–
0/100		–	0.6 (0.1) ^{ax}	1.9 (0.2) ^{by}	0.9 (0.3) ^{ax}	–	2.0 (0.2) ^{bx}	0.8 (0.4) ^{ay}	–

^{a, b, c} Different letters in the same column indicate significant differences among samples (p < 0.05)

^{x, y, z} Different letters in the same file indicate significant differences among time for a same sample (p < 0.05)

Table 10: Concentrations of active nisin for 3 days at 30 °C inside and outside hydrogel microspheres containing *L.lactis* encapsulated in stationary state. Two internal compositions of microbeads were tested: physiological water and M17 enriched with 0.5 % glucose

A/P	internal composition	active nisin (mg.mL ⁻¹)							
		inside microbeads				outside microbeads			
		day 0	day 1	day 3	day 7	day 0	day 1	day 3	day 7
100/0	physiological water	0.01 (0.01) ^{ax}	2.3(0.1) ^{ay}	—	—	—	0.6(0.3) ^a	—	—
75/25		0.02(0.01) ^{ax}	2.3 (0.4) ^{ay}	—	—	—	0.3(0.2) ^a	—	—
50/50		0.02(0.01) ^{ax}	1.8 (0.4) ^{aby}	—	—	—	1.0(0.2) ^{ab}	—	—
25/75		0.04(0.02) ^{ax}	1.6 (0.1) ^{by}	—	—	—	1.2(0.2) ^b	—	—
0/100		0.06(0.05) ^{ax}	1.4(0.3) ^{by}	—	—	—	1.7(0.2) ^c	—	—
100/0	M17 enriched with 0.5 % glucose	0.1(0.06) ^{ax}	2.5(0.2) ^{ay}	0.9(0.2) ^{az}	0.2(0.1) ^{ax}	—	0.6(0.3) ^{ax}	—	—
75/25		0.1(0.1) ^{ax}	2.9(0.2) ^{ay}	1.2(0.2) ^{az}	0.4(0.2) ^{ax}	—	0.3(0.2) ^{ax}	—	—
50/50		0.14(0.09) ^{ax}	2.8(0.3) ^{ay}	0.8(0.4) ^{az}	0.3(0.2) ^{ax}	—	1.0(0.2) ^{bx}	0.17(0.06) ^a _y	—
25/75		0.10(0.05) ^{ax}	2.6(0.2) ^{ay}	0.8(0.3) ^{az}	0.3 (0.2) ^{ax}	—	1.2(0.4) ^{bx}	0.07(0.02) ^a _y	—
0/100		0.08(0.05) ^{ax}	2.2(0.3) ^{ay}	0.6(0.3) ^{az}	0.2 (0.2) ^{ax}	—	1.7(0.2) ^{bx}	—	—

^{a, b, c} Different letters in the same column indicate significant differences among samples (p <0.05)

^{x, y, z} Different letters in the same file indicate significant differences among time for a same sample (p <0.05)

2.2.3. Antimicrobial activity

As commented above the best system to protect *L.lactis* and permit nisin release is the use of composite microbeads (A/P:75/25; internal medium: glucose-enriched M17) with LAB in exponential state. The possible antilisterial effect of this system at 30 °C was determined on TSB medium and is shown in Figure 28a. Non-encapsulated *L. lactis* was used as control. *Listeria monocytogenes* population increased from 2.8 to 7.9 log CFU.mL⁻¹ at the end of the storage period. As expected, in presence of *L. lactis* free or encapsulated a complete inhibition of *L. monocytogenes* growth was observed during all storage period. Mechanisms underlying these antimicrobial effects have not been studied, but it can be a combination of several factors such as the production of organic acids, hydrogen peroxide, enzymes, lytic agents and other antimicrobial peptides, or bacteriocins (Alzamora *et al.*, 2000). Antimicrobial properties of *L. lactis* were not limited by the system of encapsulation developed in this study. Moreover from the 5th day of storage at 30 °C reduction of *L.monocytogenes* counts was higher with microbeads. These data can certainly be explained by a difference in LAB viability. As is shown in Fig. 28b, *L.lactis* population grew immediately after the incorporation of the strain on TSB medium. No differences between non-encapsulated bacteria and microbeads were observed until 3rd day of storage. From the 5th day *L.lactis* counts decrease to achieve 8.8 and 6.9 log CFU.mL⁻¹ for encapsulated and free strain respectively. A loss of elementary nutrients in synthetic media explains these results.

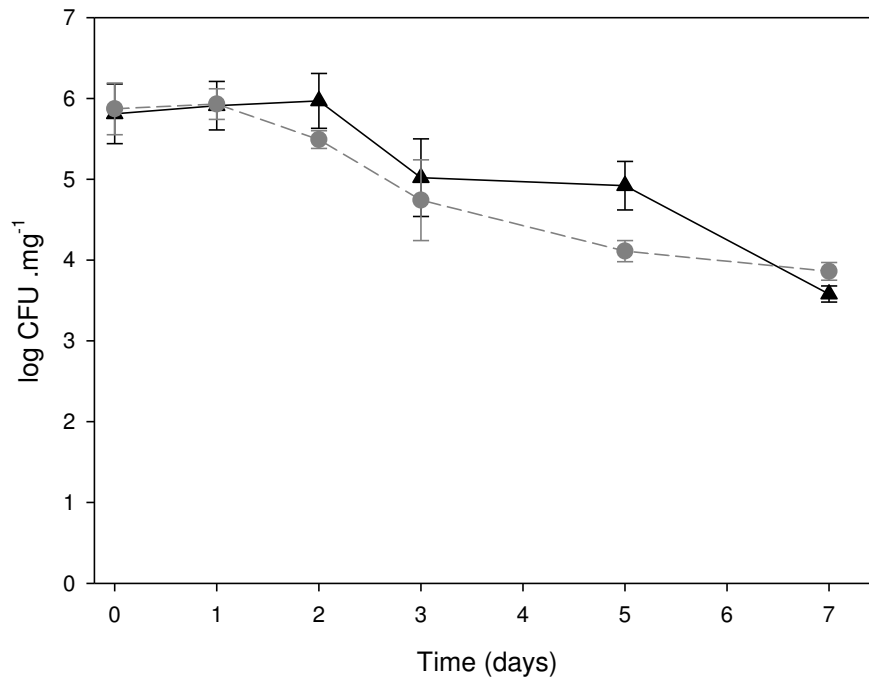


Figure 27: Survival of *Lactococcus lactis* non-encapsulated during a storage period in physiological water of 7 days at 30 °C (stationary state in solid line and exponential state in dash line). Mean values and standard deviations.

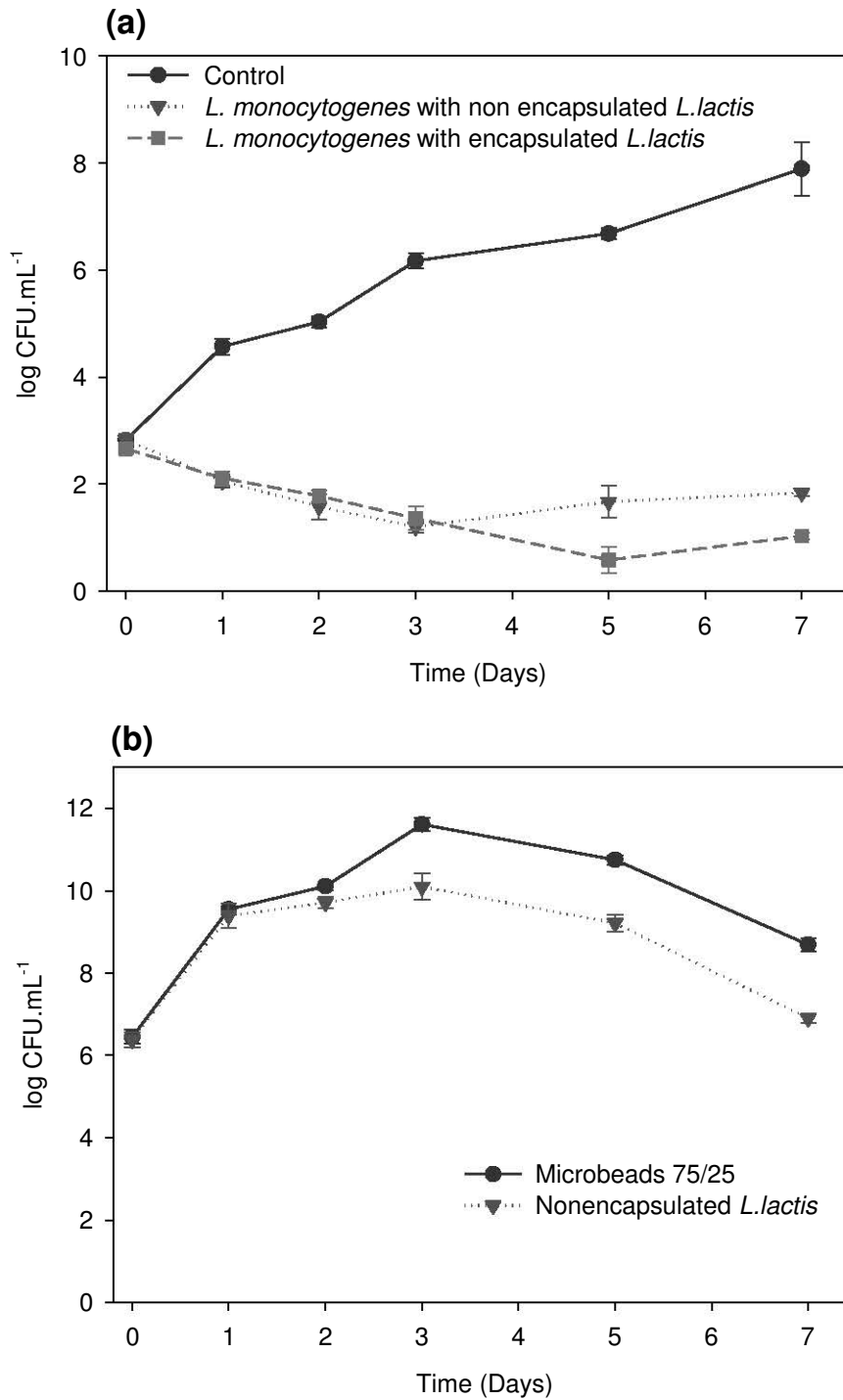


Figure 28: . Effect of *Lactococcus lactis* free or encapsulated (microbeads 75/25) on the growth of *Listeria monocytogenes* (▲ *L.lactis* non-encapsulated, ★ microbeads 75/25 and control in solid line) on TSB medium stored at 30 °C (a) and survival of *L.lactis* in contact with the culture medium (▲ *L.lactis* non-encapsulated, ● microbeads 75/25) (b). Mean values and standard deviations.

3. Conclusion

Microencapsulation of LAB producing nisin, *L. lactis* subsp. *lactis*, was performed on composite alginate / pectin hydrogel microspheres. The physical properties and the entrapped efficiency of the alginate/pectin beads are greatly affected by the biopolymers ratio used. The best mechanical properties were found for alginate/pectin: 75/25; the beads were more stable and allow the best release of nisin during the storage period. The preparation of alginate/pectin inter-penetrating network resulted in a better control of physicochemical properties of composite microbeads and potentially of the hydrogel mesh size. The physiological state of the bacteria during the encapsulation process and the composition of the microbeads (A/P ratio, enrichment of internal medium with nutrients) were determining factors for both bacteria viability and bacteriocin activity which could be related with the nutritional or cellular stress-producing effects. The best results were obtained with composite microbeads (75/25) enriched with M17 supplemented with 0.5 % glucose.

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**Chapter IV: Design of microcapsules containing
Lactococcus lactis subsp. *lactis* in alginate shell and
xanthan gum with nutrients core**

Ce chapitre présente l'encapsulation de *Lactococcus lactis* subsp. *lactis* dans des particules composées d'une membrane d'alginate et un cœur de xanthane. Les micro-perles ont été préparées avec de l'alginate pour la membrane et de la gomme xanthane enrichie en glucose enrichi M17 ou additivée d'eau physiologique dans le cœur, combinés avec *L.lactis* dans différents états physiologiques.

L'objectif de cette étude était de développer une nouvelle technique de préparation de billes qui améliorerait la survie de *L.lactis*, ainsi que l'activité antimicrobienne par rapport à *L.lactis* libre. Les capsules ont été préparées avec de l'alginate pour la fraction membranaire (1,3%) avec de l'eau physiologique et de la gomme de xanthane dans le cœur de 0,2% avec de l'eau physiologique ou de bouillon M17 stéril additionné de 0,5% de D (+) glucose. L'inoculum en bactérie dans les microsphères était de 10^5 CFU.mg⁻¹.

La caractérisation physico-chimique des microsphères a permis d'évaluer le diamètre, la sphéricité et la convexité des perles de 0 à 10 jours à 30 ° C. Toutes les microsphères ont été produites dans les mêmes conditions. On constate que le diamètre de microbilles, la sphéricité et la convexité ne présente pas de différences significatives au jour 0. Après 10 jours, la constatation est la même, ce qui signifie que ce type de capsules est plus stable et offre une bonne protection des bactéries vis-à-vis des conditions externes.

La survie et la croissance de *L.lactis* dans les microsphères pendant 10 jours à 30 ° C change de manière significative selon deux facteurs : la composition interne de la microsphère (eau physiologique ou M17 enrichi avec 0,5% de glucose), et l'état physiologique de *L.lactis*. Les résultats montrent que les conditions optimales de viabilité de la bactérie encapsulée sont : un état de croissance exponentiel et 0.5% glucose dans le milieu M17 avec la gomme de xanthane.

L'activité de la nisine a été comparée entre les bactéries encapsulées et libres en phase exponentielle dans de l'eau physiologique pendant 10 jours à 30 ° C. Il a été observé que

l'activité de la nisine est meilleure dans le cas de *L. lactis* encapsulée en phase exponentielle et avec un milieu nutritif de M17 enrichi avec 0.5% de glucose pendant 10 jours. En effet, il a été montré que la réduction de la population de *L. monocytogenes* était plus élevée avec *L. lactis* encapsulée qu'avec *L. lactis* libre.

En conclusion, les meilleurs résultats ont été obtenus avec des microsphères à membrane d'alginate et cœur de xanthane avec 0.5% glucose dans un milieu M17 et pour *L. lactis* encapsulée en phase exponentielle.

Abstract

Aqueous-core microcapsules with sodium alginate (SA) hydrogel in membrane and xanthan gum (XG) in core were prepared using ionotropic gelation method to encapsulate bacteriocin-producing lactic acid bacteria (LAB). In this study LAB were immobilized in microcapsule membrane. XG was applied to reinforce SA microcapsules. Molecular interaction between SA and XG in the microcapsules was investigated using FTIR spectroscopy. Microcapsules morphology and mechanical properties were examined. The impact of an enrichment with nutrients of core (M17 broth supplemented with 0.5% glucose vs physiological water) and physiological state of *Lactococcus lactis* during encapsulation step (exponential vs stationary state) was studied on *L. lactis* survival, nisin release. Furthermore, the antimicrobial effectiveness of the best system to preserve bacterial survival and permit nisin release was studied against *Listeria monocytogenes*.

No differences were observed in terms of mechanical properties among studied systems. FTIR spectroscopy confirmed the establishment of possible hydrogen bonding between XG hydroxyls groups and SA carboxylate groups which could modify microcapsules release properties. Microcapsules with *L. lactis* in exponential state encapsulated in SA membrane and aqueous-core based on XG with nutrients gave the best results. At 30 °C, a complete inhibition of *L. monocytogenes* growth throughout the storage period was observed for these microcapsules. These microcapsules could be used for future applications in food preservation and particularly in food packaging. Novel bioactive films based on biopolymers and enriched with *L. lactis* in alginate/xanthan gum core-shell microcapsules could be designed.

Keyword: Alginate, Xanthan gum, Lactococcus lactis, Antilisterial activity

1. Introduction

Application of lactic acid bacteria (LAB) as a biopreservation strategy has increasing interest in the last decades. LAB are considered as GRAS (Generally Recognized As Safe) and can inhibit the growth of different bacteria, yeasts and fungi, through the production of organic acids, hydrogen peroxide, enzymes, defective phages, lytic agents and antimicrobial peptides, or bacteriocins (Alzamora et al., 2000). Among pathogens present in foodstuffs, *Listeria monocytogenes* remains one of the major problems particularly in dairy products. Previous studies have already proved the antilisterial efficacy of LAB in model systems (Antwi et al., 2008), in dairy products (Liu et al., 2008), in sea-food products (Concha-Meyer et al., 2011), as well as in meat products (Maragkoudakis et al., 2009). To guarantee food safety, the incorporation of LAB into food packaging appears an interesting novel approach. But some recent studies reported problems of LAB viability (Sánchez-González et al., 2013 ; Sánchez-González et al., 2014). The use of encapsulation techniques to protect LAB before their addition in bioactive films could be an interesting approach to limit this phenomenon. Indeed, microencapsulation methods permit the entrapment of microbial cells within particles based on different materials and their protection against non-favorable external conditions (Champagne & Kailasapathy, 2008; Zuidam & Shimoni, 2010). Different factors such as encapsulation method, type and concentration of materials used, particle size and porosity or type of microparticles (bead, capsule, composite, coating layer..) affect effectiveness of the bacterial protection (Ding & Shah, 2009). Several biopolymers were studied for encapsulation: alginate, pectin, k-carrageenan, xanthan gum, gellan gum, starch derivatives, cellulose acetate phthalate, casein, whey proteins and gelatin. Alginate has been widely used as microencapsulation material, as it is non-toxic, biocompatible, and cheap (Jen et al., 1996; Léonard et al., 2013; Léonard et al., 2014). Sodium alginate (SA) composition is variable and consists in homopolymeric and heteropolymeric blocks alternating 1,4-linked β -D-mannuronic acid (M)

and α -L guluronic acid (G) residues (Pawar & Edgar, 2012). Physical properties of alginate are dependent of the composition, sequence and molecular weight (Pawar & Edgar, 2012). Gel formation is driven by interactions between G-blocks, which associate to form firmly held junctions due to divalent cations. In addition to G-blocks, MG blocks also participate by forming weaker junctions (Pawar & Edgar, 2012). Some studies focus on the interest on design composite systems on associating several biopolymers such as alginate and xanthan gum to control active components release (Wichchukit et al., 2013). Xanthan Gum (XG) is an extracellular anionic polysaccharide secreted by *Xanthomonas campestris*. It is a complex polysaccharide consisted in a primary chain of β -D-(1,4)-glucose backbone, which has a branching tri-saccharide side chain comprised of β -D-(1,2)-mannose, attached to β -D-(1,4)-glucuronic acid, and terminates in a β -D-mannose (Elçin, 1995; Goddard & Gruber, 1999). Recently, XG has been used to combine with SA in beads to preserve LAB viability and modulate release properties. This could be due to the molecular interaction between SA and XG, which led to the formation of a complex matrix structure (Fareez et al., 2015 and Pongjanyakul and Puttipipatkachorn, 2007).

The aim of the present study was to develop novel SA-XG microspheres that can enhance stability of *L.lactis* and release of nisin during storage for future food packaging applications. LAB were usually present inside beads or in the core of capsules. One of the originalities of this study was to immobilize bacteria in SA membrane of the capsule and to use the core as a nutrient pool to permit a gradual bacterial growth. Physico-chemical properties of microcapsules were studied. The effect of bacteria physiological state during encapsulation step (exponential or stationary phase) and a possible enrichment of aqueous-core with nutrients (M17 supplemented with 0.5 % glucose vs physiological water) on bacterial survival, nisin release and antilisterial activity was studied.

2. Results and Discussion

2.1. Physico-chemical characterization of microcapsules

2.1.1. Shape and size

Microscopic image of freshly prepared SA-XG microcapsules was presented on Fig. 29. Capsule was fairly spherical and the aqueous-core was centered.

Microcapsules size, sphericity and convexity at day 0 and after 7 days at 30 °C were reported on Table 11. For a given procedure of capsules production (given nozzle diameter, vibration frequency, extrusion flow rate), the average diameter was demonstrated dependent of the viscosity of injected fluid (Cellesi et al., 2004). In this study viscosity was set to obtain rather spherical particles. Results of sphericity and convexity were in accordance with microscopic observations and indicated that just-prepared capsules were rather spherical and with an irregular surface. Therefore, a sphericity value “1” correspond to a perfect sphere and a particle with sphericity close to “0” is highly irregular. Convexity provides information about the roughness of the particle. Convexity values are between 0 and 1. A particle with smooth edges has a convexity value of one whereas a particle with irregular ones has a lower convexity (Burgain et al., 2011). All parameters measured remained constant during the storage period. Microcapsules thus remained stable under conditions tested in this study.

Table 11 : Size, sphericity and convexity of microcapsules at day 0 and after a storage period in physiological water of 10 days at 30 °C.

Time (days)	Size D[4,3] (µm)	Sphericity	Convexity
0	413(10) ^a	0.6(0.2) ^a	0.23(0.02) ^a
10	419(7) ^a	0.6(0.2) ^a	0.27(0.02) ^a

^{a,b,c} Different letters indicate significant differences between the various samples among the same column (p <0.05)



Figure 29: Microscopic image of single alginate-xanthan gum microcapsule

2.1.2. Mechanical stability

SA capsules are sensitive to deformations that may lead to their rupture or to undesirable early release of their contents. In order to evaluate the mechanical stability of the prepared systems, individual microcapsule was compressed between two parallel plates. Fig. 30 reports the plot of the normal force (N) versus the displacement (μm) for SA-XG microcapsules prepared with physiological water and M17 enriched with glucose. The microcapsules did not show a rupture point (a maximum peak force followed by a dramatic force decrease), even when the capsules were compressed down to a thickness of around $5\ \mu\text{m}$ from an original diameter of $500\ \mu\text{m}$. The compression curves showed the same profiles which means that the aqueous core composition of the microcapsules did not influence the compression profile of the microcapsules. Under compression, the alginate physical cross-links could restructure in a more dense fashion, releasing the water in excess and resulting in a volume reduction (Cellesi et al., 2004). The water expulsion could be also accentuated due to the low speed used during the compression experiments ($10\ \mu\text{m}\ \text{s}^{-1}$) which might be lower than the water release kinetic from the squeezed microcapsules.

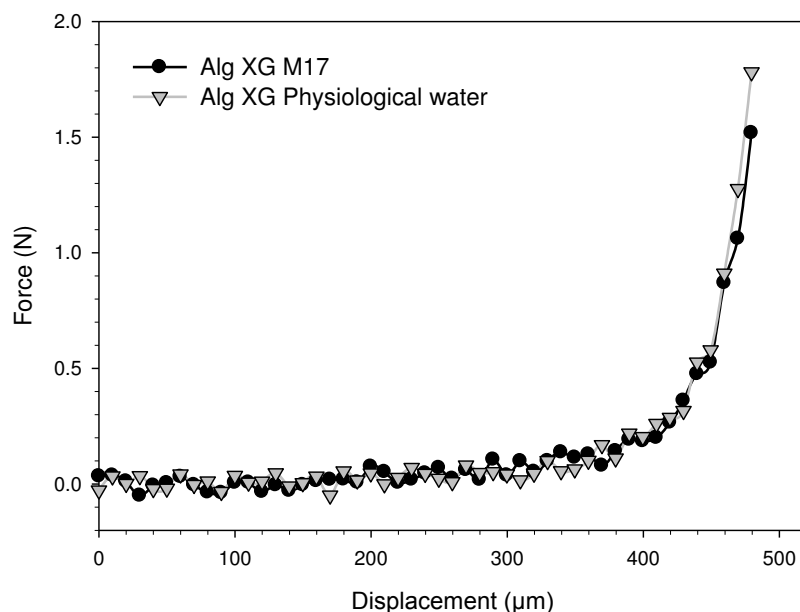


Figure 30: Typical force-displacement curves of alginate-xanthan microcapsules prepared with M17 and physiological water

2.1.3. FTIR spectroscopy

The FTIR spectra of freeze-dried SA-XG solutions and microcapsules are shown in Fig. 31. All spectra displayed a band between 3000 and 3700 cm^{-1} (OH stretching) followed by a small band (3000-2850) due to CH stretching.

FTIR spectra of SA (a) showed two characteristic peaks around 1595 and 1408 cm^{-1} , indicating the stretching of COO^- (asymmetric) and COO^- (symmetric), respectively. The band at 1020 cm^{-1} is an antisymmetric stretch (C-O-C) given by the guluronics units (Pereira et al., 2003).

The FTIR spectrum of xanthan gum (b) shows two carbonyl peaks (C=O) at 1725 cm^{-1} corresponding to acetate groups of an inner mannose unit and at 1600 cm^{-1} , the characteristic band of carboxylate of pyruvate group and glucuronic acid (Hamcerencu et al., 2007).

The reticulation of alginate with calcium cations caused a decrease in intensity of COO⁻ stretching peaks, and a decrease in intensity of 1031 cm⁻¹ peak (c). This indicated that an ionic bonding between calcium ion and carboxyl groups of SA and a partial covalent bonding between calcium and oxygen atom of ether groups, respectively. The incorporation of XG into calcium alginate microcapsules (d) did not show significant modifications. However, SA-XG microcapsules spectra (d) showed a decrease of peak intensity of the carboxylate bands. This observation could be related to potential hydrogen bonding between alginate carboxylate groups and XG hydroxyls groups (Pongjanyakul & Puttipipatkachorn, 2007). Otherwise, the freeze-drying step of microcapsules before the FTIR study could also initiate or reinforce the hydrogen bonding between the two polymers.

As described in the literature, the barriers capsules could be obtained by coating technique in which negative charge polymers, as “alginate”, are coated by positively charged polymers, such as “chitosan”. Coating is used to enhance gel stability (Smidsrød et al., 1990 and Kanekanian, 2013) and provide a better barrier to cell release (Gugliuzza, 2014; Zhou, Martins, Groboillot, Champagne, & Neufeld, 1998). The reaction of the biofunctional molecule with the membrane resulted in bridge formation. The length of the bridge depended on the type of cross-linking agent (Hyndman, Groboillot, Poncelet, Champagne, & Neufeld, 1993). Nevertheless, in the present study, FTIR spectra of SA-XG microcapsules did not exhibit the characteristics bands of XG which could be related to potential ionic bonding formation between XG and calcium ions. The low amount of XG compared to the SA concentration could explain this unapparent change in the FTIR spectra.

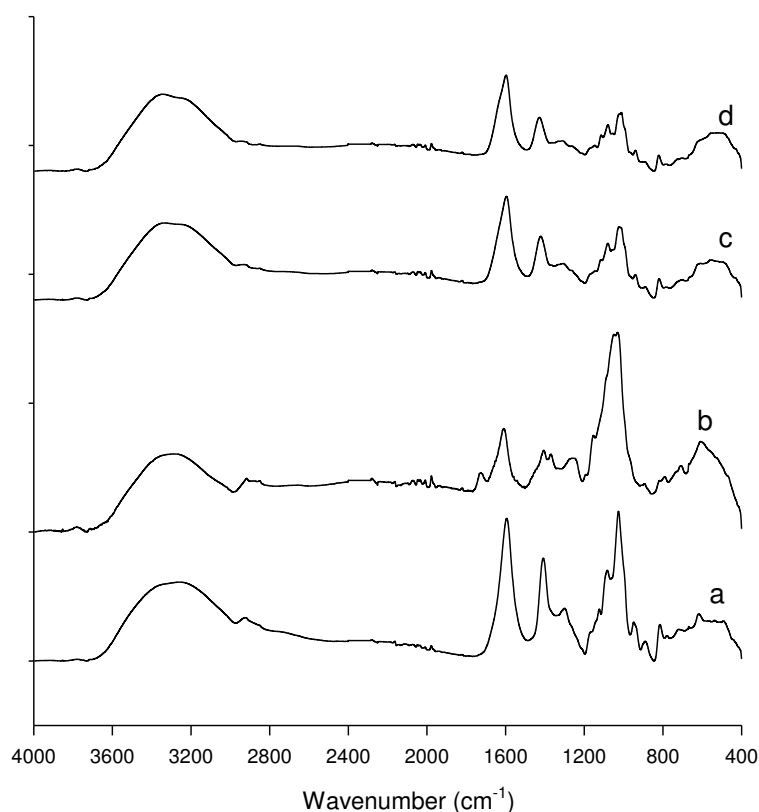


Figure 31: FTIR spectra of freeze-dried (a) alginate solution, (b) xanthan gum solution, alginate microbeads (c) and alginate xanthan microcapsules (d).

2.2. Encapsulation of *L.lactis*

2.2.1. *L.Lactis* survival

The survival of *L.lactis* inside and outside of two types of microcapsules was studied in physiological water for 10 days at 30°C (Fig. 32). The aim of this part was to study if a possible enrichment of microcapsules core with nutrients (M17 supplemented with 0.5 % glucose) and the physiological state of the strain during microcapsule production were key factors to preserve viability of *L.lactis*. Non-encapsulated *L.lactis* was used as control (data not presented). During the storage period a significant decrease of bacterial counts were observed for bacteria in exponential or in stationary state at the beginning of the assay to achieve after 10 days at 30 °C

3 and 1.8 log CFU.mg⁻¹ respectively. The lack of nutrients can certainly explain these results, since physiological water permit only to control osmotic pressure and to avoid cellular lysis.

Immobilization of *L.lactis* in SA membrane seems preserve bacterial viability in physiological water. Although bacterial population inside capsules decreased significantly only after 3 days at 30 °C at the end of the storage period microbial counts remains above 3 log CFU.mg⁻¹ regardless what physiological state of the strain at the beginning of assay and core composition (Fig 32 a, b). Addition of nutrients within microbeads led to little differences in terms of *L. lactis* population. This is certainly due to a rapid release of some nutrients (small molecules) to the storage medium. Indeed, recent studies reported that the pore size of hydrogel beads may range from around 5 to 200 nm depending on their composition and preparation (Zeeb et al., 2015 and Gombotz and Wee, 1998). The combination of the supply of nutrients in core and the encapsulation of LAB during exponential phase gave the best results in terms of viability (Fig 32a); in these conditions *L. lactis* counts were maintained to 5 log CFU.mg⁻¹ after 10 days at 30 °C.

Concerning bacterial counts outside capsules, in physiological water, a significant increase during the beginning of the storage period was observed whatever the composition of the microcapsules. A maximum level of 4.5 log CFU mg⁻¹ was achieved after 5 days of microcapsules storage at 30 °C. When capsules were placed into physiological water, bacteria adsorb on surface or present on alginate membrane were certainly progressively released to external medium. From the 5th day of storage, *L.lactis* population started to decrease significantly to achieve 3.5 log CFU.mg⁻¹ at the end of the storage period.

Polymers choice and structure of microcapsules were key factors but results of this study focused on the importance of two supplementary parameters, physiological state of LAB during the encapsulation step and the possible enrichment of capsules with nutrients. These factors

affect certainly cellular stress and therefore bacterial survival. Microcapsules prepared with *L.lactis* in exponential state and enriched with nutrients in core (M17 supplemented with 0.5 % glucose) present best results to maintain *L.lactis* counts inside and outside capsules.

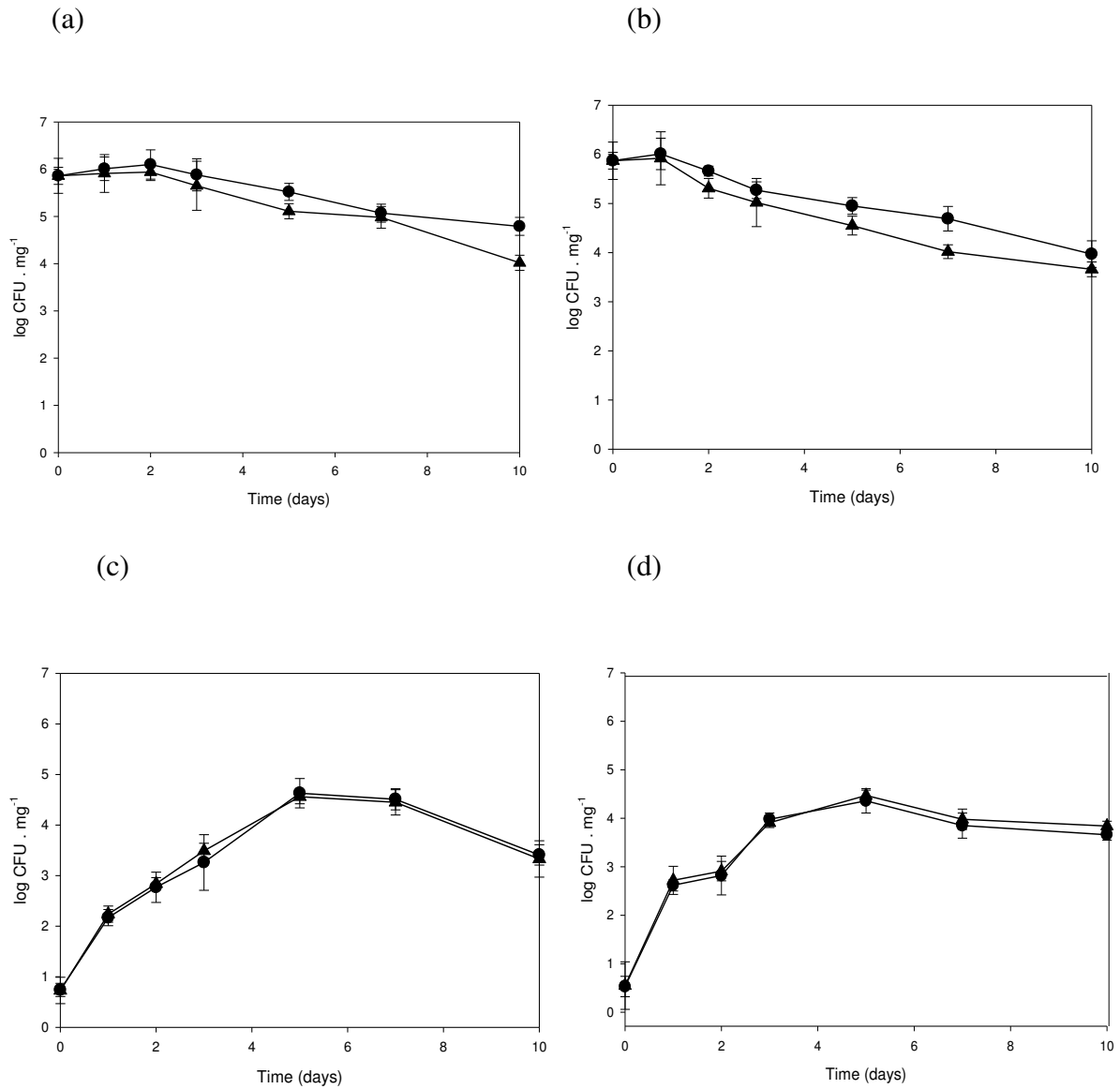


Figure 32: Survival of *Lactococcus lactis* inside (a,b) and outside (c,d) microcapsules, during a storage period in physiological water of 10 days at 30 °C (▲physiological water, ● M17 supplemented with glucose) (a,c) *L.lactis* encapsulated in exponential state (b, d) *L.lactis* encapsulated in stationary state. Mean values and standard deviations.

2.2.2. Nisin activity

Nisin activity inside and outside microcapsules was determined for different systems in physiological water at 30°C for 10 days (Table 12). As observed for bacterial survival, bacterial physiological state as well as addition of nutrients in core were key factors to optimize nisin activity and consequently antimicrobial properties of microcapsules.

Maximum concentration of active nisin inside capsules was detected after 1 day of storage at 30 °C independently of the aqueous-core composition and bacterial physiological state. A significant decrease in the amount of active nisin was then observed. Addition of nutrients in core reduces this phenomenon, a slight concentration of active bacteriocin was reported at the end of the storage period. Previous studies reported equally changes in term of bacteriocin concentration with the composition of the nutrient broth but for non-encapsulated bacteria (Parente & Ricciardi, 1999).

Bacterial physiological state of *L.lactis* during encapsulation step appears equally as an important factor. When LAB was encapsulated in stationary state, a small concentration of active nisin was detected inside microcapsules at day 0 because nisin was already produced by the bacteria before encapsulation. Conversely, when *L.lactis* was encapsulated in exponential phase, no nisin was detected initially in capsules. Therefore the physiological state of the LAB at the time of encapsulation impacts the initial concentration of active nisin in microcapsules.

Concerning release of active compound, it is necessary to wait one day of storage to detect a significant concentration of active nisin outside microcapsules, in physiological water. This amount gradually increases to achieve a maximum after one or three days at 30 °C when *L.lactis* was encapsulated in stationary and exponential state respectively. Production of nisin occurs during bacterial growth. When bacteria were encapsulated in stationary phase, cell growth was completed. A high concentration of nisin has been released but a significant part is certainly

remained adsorbed on the surface of bacterial cells. This fraction was encapsulated with bacteria and quickly released outside microcapsules. After 10 days at 30 °C the presence of active nisin in the solution of NaCl used for the storage of microcapsules was detected only for one of the tested systems: capsules with alginate membrane and aqueous-core based on nutrients (M17 enriched with 0.5 % glucose) prepared using bacteria in exponential state.

To conclude with this part, to optimize an encapsulation system it is necessary to take into account two parameters that influence bacterial survival and production of bacteriocin, bacterial physiological state during the encapsulation process and the possible addition of nutrients in the system. These factors could be related with nutritional or cellular stress-producing effects. Microcapsules with *L.lactis* in exponential state encapsulated in alginate membrane and aqueous-core based on xanthan gum with nutrients (M17 enriched with 0.5 % glucose) gave the best results.

Table 12 : Concentrations of active nisin for 10 days at 30 °C inside and outside alginate-xanthan microcapsules containing L.lactis encapsulated in stationary or exponential state. Two internal compositions of microcapsules were tested: physiological water and M17 enriched with 0.5 % glucose. Mean values and standard deviations.

bacterial physiologic al state	core composition	active nisin (mg.mL ⁻¹)									
		inside microcapsules					outside microcapsules				
		day 0	day 1	day 3	day 7	day 10	day 0	day 1	day 3	day 7	day 10
exponential	physiological water	-	1.4(0.2) ^{aw}	0.2(0.2) ^{ax}	0.01(0.02) ^{ax}	-	-	0.2(0.2) ^{aw}	0.6(0.3) ^{aw}	-	-
	M17 enriched with 0.5 % glucose	-	2.0(0.2) ^{bw}	0.8(0.3) ^{bx}	0.6(0.2) ^{bx}	0.22(0.03) ^{a_y}	-	0.4(0.2) ^{aw}	0.9(0.4) ^{aw}	0.2(0.2) ^{aw}	0.05(0.02) ^{ax}
stationary	physiological water	0.3(0.2) ^{aw}	1.2(0.2) ^{ax}	0.2(0.2) ^{aw}	-	-	-	0.3(0.2) ^{aw}	0.12(0.08) ^{b_x}	-	-
	M17 enriched with 0.5 % glucose	0.3(0.2) ^{aw}	1.5(0.2) ^{ax}	0.6(0.2) ^{bw}	0.14(0.02) ^{cy}	0.01(0.02) ^{b_z}	-	0.7(0.3) ^{aw}	0.6(0.2) ^{aw}	-	-

^{a, b, c} Different letters in the same column indicate significant differences among samples (p <0.05)

^{x, y, z} Different letters in the same file indicate significant differences among time for a same sample (p <0.05)

2.2.3. Antimicrobial activity

As discussed above, the best system to preserve bacterial survival and permit nisin release was the use of microcapsules with *L.lactis* in exponential state encapsulated in SA membrane and aqueous-core based on XG with nutrients (M17 enriched with 0.5 % glucose). Antilisterial properties of this system were determined at 30°C on synthetic medium, TSB (Fig. 33a). Non-encapsulated *L.lactis* was used as a control. *Listeria monocytogenes* population increased from 2.8 to 7.9 log CFU.mL⁻¹ at the end of the storage period. A clear inhibition of *L. monocytogenes* growth was observed in presence of *L.lactis* free or encapsulated. Antimicrobial properties of *L.lactis* were not limited by the system of encapsulation developed in this study.

Moreover from the 3rd day of storage at 30°C reduction of *L.monocytogenes* counts was higher with *L.lactis* encapsulated than with free *L.lactis*. These data can certainly be explained by a difference in LAB viability. As is shown in Fig. 33b, *L.lactis* population grew immediately after the incorporation of the strain on TSB medium. No differences between non-encapsulated bacteria and encapsulated bacteria were observed until 2nd day of storage. From the 3rd day *L.lactis* counts decrease to achieve at the end of the storage period 8.6 and 6.6 log CFU.mL⁻¹ for encapsulated and free strain respectively. A loss of elementary nutrients in synthetic media explains these results. Considering the effect of the size of the capsule on antilisterial activity, the smaller beads were more effective than larger beads due to higher surface/volume ratio, as previously observed by Anal, Stevens, and Remunan-Lopez (2006) for ampicilin/chitosan capsules.

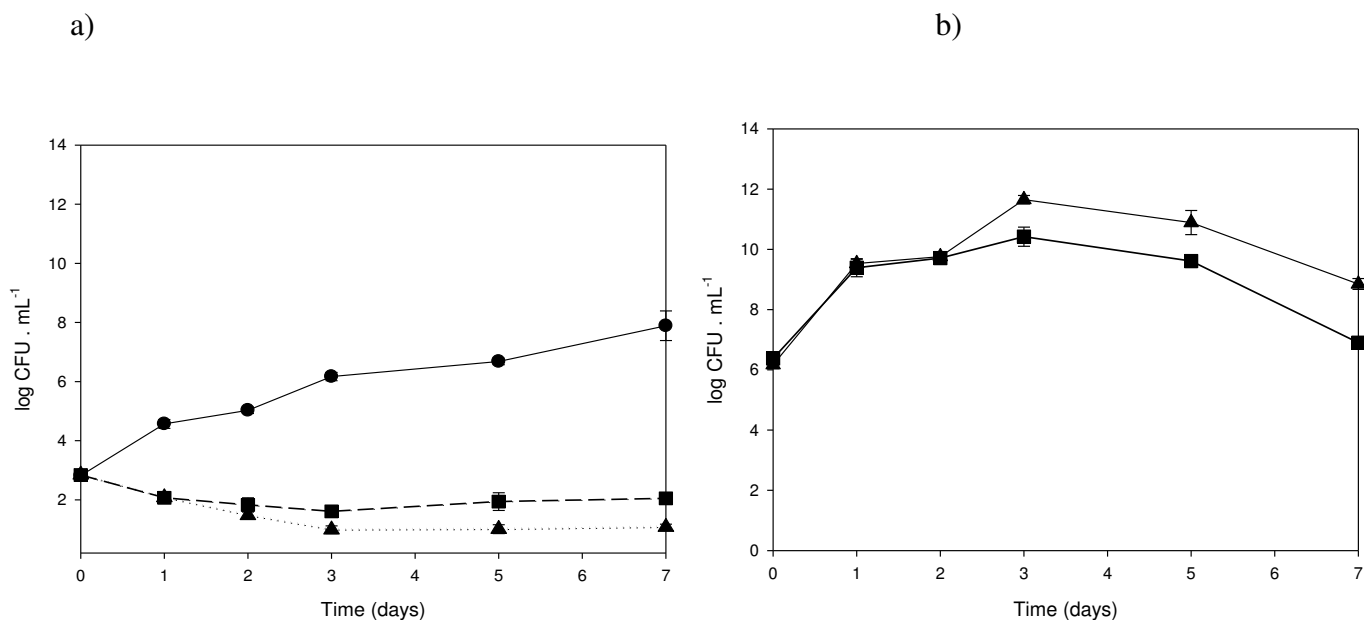


Figure 33: Effect of *Lactococcus lactis* free or encapsulated in exponential state (alginate-xanthan microcapsules with internal medium prepared with glucose-enriched M17) on the growth of *Listeria monocytogenes* (□ *L.lactis* non-encapsulated, ▲ alginate-xanthan microcapsules and control in solid line) on TSB medium stored at 30°C (a) and survival of *L.lactis* in contact with the culture medium (■ *L.lactis* non-encapsulated, ▲ alginate-xanthan microcapsules) (b). Mean values and standard deviations.

3. Conclusion

SA/XG core-shell microcapsules were developed for immobilization of *L. lactis* on membrane. Microcapsules present a plastic behavior and no differences were observed in terms of mechanical properties among studied systems. The aqueous core composition of the microcapsules did not affect SA network stability. Addition of XG caused a change in matrix structure of microcapsules membrane by establishment of potential hydrogen bonding between XG hydroxyls groups and SA carboxylate groups. This certainly leads to better LAB protection

and change nisin release. The physiological state of the bacteria during the encapsulation process and the enrichment of aqueous-core with nutrients (M17 supplemented with 0.5% glucose) were determining factors for both bacteria viability and bacteriocin activity.

Microcapsules with *L. lactis* in exponential state encapsulated in alginate membrane and aqueous-core based on xanthan gum with nutrients (M17 enriched with 0.5% glucose) gave the best results in terms of bacterial viability and nisin activity. These microcapsules allow a complete inhibition of *L. monocytogenes* growth for 7 days at 30 °C. Antimicrobial properties of *L. lactis* were not limited by the system of encapsulation developed in this study. It could be interesting to design novel bioactive food packaging based on biopolymers films enriched with these SA/XG core-shell microcapsules.

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Chapter V: Physical properties and antilisterial activity of bioactive films containing alginate/pectin composite microbeads with entrapped *Lactococcus lactis* subsp *lactis*.

Physical properties and antilisterial activity of bioactive films containing alginate/pectin composite microbeads with entrapped *Lactococcus lactis* subsp *lactis*.

Abstract

Novel bioactive films were developed from the incorporation of *Lactococcus lactis* subsp. *lactis* into polysaccharide films. Two different biopolymers were tested, cellulose derivative (hydroxypropylmethylcellulose) and corn starch. Lactic acid bacteria free or encapsulated in alginate/pectin composite microbeads were added directly to the film forming solution and films were produced by casting. Impacts of the incorporation of *L. lactis* into the biopolymer matrix, the water vapour permeability (WVP), oxygen permeability (OP), optical and mechanical properties of the dry films were shown. Beads incorporation reduced the differences between both film systems. Furthermore, the antimicrobial activity of bioactive films against *Listeria monocytogenes* was demonstrated in synthetic medium with higher activity for both film with capsuled, compared to free *L. lactis*.

Keywords: biopolymer, bioactive films, microbeads, starch, hydroxypropylmethylcellulose, mechanical properties.

1. Introduction

Lactic acid bacteria (LAB) are traditionally used to provide taste, texture and increase the nutritional value of fermented foods such as dairy products (yoghurt, cheese), meat products, as well as some vegetables. However, a large amount of research has been focused on the great potential of LAB use for food preservation. Studies have shown that LAB can inhibit the growth of different microorganisms, including bacteria, yeasts and fungi, through the production of organic acids, hydrogen peroxide, enzymes, defective phages, lytic agents and antimicrobial peptides, or bacteriocins (Alzamora et al., 2000).

During the last years, innovative bioactive films enriched with LAB have been developed (Gialamas et al., 2010; Sánchez-González et al., 2013; Sánchez-González et al., 2014). Among biopolymers used as support for LAB, cellulose derivatives appeared as remarkable film forming compounds. They are not only biodegradable, odorless and tasteless (Krochta and Mulder-Johnston, 1997) but they also exhibit good barrier properties against lipids, oxygen and carbon dioxide at low and intermediate relative humidity (Nispero-Carriedo, 1994). Hydroxypropylmethyl cellulose (HPMC) has been also used for its good film forming properties and mechanical resistance. The third interesting polysaccharide used in active packaging is starch. This biopolymer is a renewable resource, inexpensive (compared with other compounds) and widely available (Lourdin, Della Valle & Colonna, 1995).

However, one of the major problems encountered was the decrease of the film's antimicrobial activity throughout time due to LAB viability problems (Sánchez-González et al., 2013; Sánchez-González et al., 2014). To limit this problem and increase films effectiveness, encapsulation techniques appeared as an interesting approach. Indeed, microencapsulation methods permitted the entrapment of microbial cells within particles based on different materials and their protection against non-favorable external conditions (Champagne & Kailasapathy, 2008; Zuidam & Shimoni, 2010). Different factors such as encapsulation method,

type and concentration of materials used, particle size and porosity or type of microparticles (bead, capsule, composite, coating layer..) were show to affect effectiveness of bacterial protection (Ding & Shah, 2009). Alginate has been widely used as microencapsulation material as it is non-toxic, biocompatible, and cheap (Jen et al., 1996; Léonard et al., 2013; Léonard et al., 2014). Alginate consists in homopolymeric and heteropolymeric blocks alternating 1,4-linked β -D-mannuronic acid (M) and α -L guluronic acid (G) residues in which the G units form crosslinks with divalent ions, to produce “egg-box” model gels (Pawar & Edgar, 2012). Studies have reported that alginate can form strong complexes with other natural polyelectrolytes such as pectin (also a polyuronate) by undergoing chain–chain association and forming hydrogels upon addition of divalent cations (e.g., Ca^{2+}) (Fang et al., 2008; Pillay & Fassihi, 1999a), improving mechanical and chemical stability of alginate beads, and consequently improving their encapsulation effectiveness (Pillay & Fassihi, 1999b).

The aim of the present paper was to evaluate how HPMC and corn starch films were affected by the incorporation of *L. lactis*, free or encapsulated in alginate/pectin composite microbeads, through the analysis of different physical properties (water vapor barrier, oxygen permeability, mechanical and optical properties) as well as their antilisterial impact.

2. Results and discussion

2.1. Physico-chemical properties

Properties of films equilibrated at 5°C and 75% RH and are reported in Table 1. Globally, properties of HPMC films were slightly affected by incorporation of capsules and/or bacteria while starch films were dramatically modified (huge increase of elongation properties, strong reduction of EM and TS). With entrapped bacteria, properties of both films became similar except for the oxygen barrier properties which remained highly lower in the case of starch film. The WVP values of corn starch films were in the range of those reported by Greener and Fennema (1989). The slight differences can be attributed to minor changes in the experimental

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conditions as temperature, RH gradient, kind and amount of plasticizer, etc... It was verified that biopolymer films are highly permeable to water vapor, which is coherent with the hydrophilic nature of polysaccharides (Han and Gennadios, 2005). Under these experimental conditions, significant differences were observed in WVP values between corn starch and HPMC films. This high WVP is of high interest to allow mass transport through the film and nisin activity for food safety application.

Table 1. Effect of the incorporation of lactic acid bacteria (*Lactococcus lactis*) entrapped in microcapsules (C) and microbeads (B) on film mechanical properties (Elongation, Tensile Strength, Elastic Modulus), water vapor permeability, oxygen permeability, moisture content and thickness of biopolymer films (HPMC & STarch) equilibrated at 5°C and 75% relative humidity. Mean values and standard deviation..

Film	E (%)	TS (MPa)	EM (MPa)	WVP (g.µm.m ⁻² .j ⁻¹ .kPa ⁻¹)	OP (cc.m.Pa ⁻¹ .s ⁻¹) x 10 ⁷	Moisture content (g water. g film ⁻¹)	Thickness (µm)
HPMC	57 (7) ^a	24 (4) ^a	524 (45) ^a	2.15 (0.11) ^a	46 (3) ^a	0.158 (0.002) ^a	159 (6) ^a
HPMC+C	41 (7) ^b	24 (3) ^a	561 (26) ^a	2.02 (0.11) ^a	51 (8) ^a	0.162 (0.002) ^a	210 (4) ^b
HPMC+B	58 (7) ^a	25.5 (3) ^a	473 (51) ^a	2.25 (0.13) ^a	43 (5) ^a	0.153 (0.007) ^a	153 (8) ^a
HPMC+C+B	34 (6) ^{bd}	18.0 (4) ^a	447 (34) ^a	2.22 (0.16) ^a	55 (7) ^a	0.154 (0.006) ^a	205 (3) ^b
ST	3.3 (0.2) ^c	20 (2) ^a	962 (59) ^b	3.05 (0.11) ^b	2.50 (0.14) ^b	0.166 (0.016) ^b	123 (7) ^c
ST+C	3.6 (0.3) ^c	12 (4) ^b	615 (100) ^c	2.51 (0.11) ^c	2.07 (0.06) ^c	0.242 (0.006) ^c	124 (4) ^c
ST+B	30.6 (1.8) ^d	7.1 (0.5) ^c	298 (58) ^d	3.20 (0.11) ^b	2.3 (0.2) ^b	0.130 (0.002) ^d	122 (4) ^c
ST+C+B	31 (2) ^d	6.0 (0.4) ^d	280 (62) ^d	2.42 (0.12) ^c	1.91 (0.13) ^c	0.273 (0.008) ^c	125 (7) ^c

^{a, b, c, d} Different letters in the same column indicate significant differences among formulations (p <0.05).

The optical properties of the films were evaluated through their color and transparency since these properties have a direct impact on the appearance of the coated product.

Film transparency was evaluated through the internal transmittance, Ti (0-1, theoretical range). An increase in Ti can be assumed as an increase in transparency (Hutchings, 1999). The spectral distribution of Ti (400–700 nm) is shown in Figure 1.

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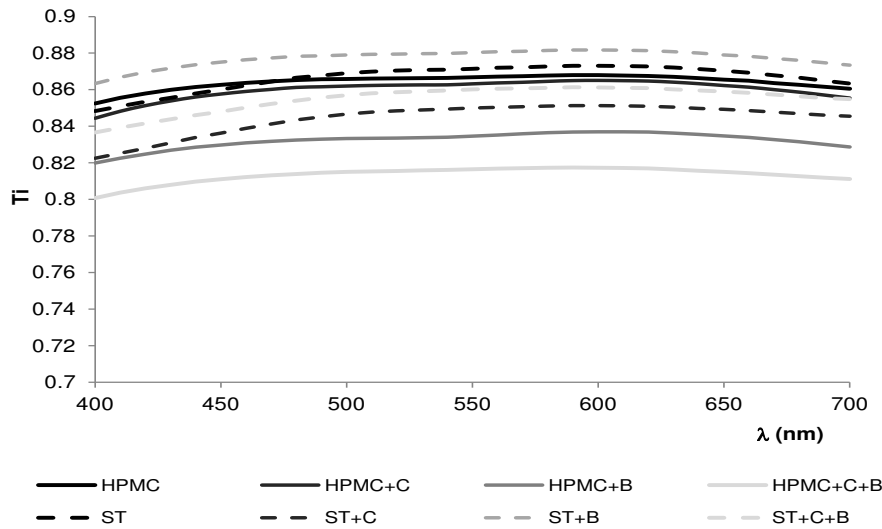


Figure 34. Spectral distribution of internal transmittance (Ti) of films equilibrated at 5 °C and 75 % RH.

The main impact was observed when both capsules and bacteria were added in the film where the transparency slightly decrease. All systems remained high transparent independently of the capsules or bacteria content. This properties, confirmed by the table 2, would also help easy applications of the films as a packaging materials.

Table 2. Lightness (L*), chrome (C*ab), hue (h*ab) and whiteness index (WI) of biopolymer films equilibrated at 5°C and 75% relative humidity. Mean values and standard deviation.

Film	L*	(C* _{ab})	(H* _{ab})	WI
HPMC	81 (3) ^a	1.8 (0.6) ^a	104 (3) ^a	73 (3) ^a
HPMC+C	79.1 (1.9) ^a	2.3 (1.2) ^a	99 (2) ^a	72 (2) ^a
HPMC+B	68.3 (0.6) ^b	0.8 (0.4) ^a	103.0 (1.5) ^a	68.2 (0.6) ^b
HPMC+C+B	63 (2) ^c	1.2 (0.2) ^a	103 (2) ^a	63 (2) ^c
ST	85.7 (1.3) ^d	7.3 (0.7) ^b	103.2 (1.8) ^a	83.9 (0.9) ^d
ST+C	73 (2) ^e	6.8 (1.9) ^b	102.4 (1.3) ^a	80 (3) ^d
ST+B	86 (3) ^d	5.0 (1.2) ^b	99.3 (1.9) ^a	86 (2) ^d
ST+C+B	73 (4) ^e	5.2 (1.7) ^b	101.8 (1.2) ^a	82 (2) ^d

^{a, b, c, d, e} Different letters in the same column indicate significant differences among formulations (p < 0.05).

2.2. FTIR analysis

FTIR spectra of freeze-dried (A/P:75/25) microbeads, preconditioned starch and HPMC films before and after microbeads incorporation are shown in Fig.2.

As discussed in our previous study, FTIR spectrum of A/P microbeads, displayed typical bands of alginate and pectin biopolymers (Fig. 2a). The band 1590-1600 cm^{-1} is related to antisymmetric stretch of C-O of alginate and pectin carboxylate groups. The peak at 1413 cm^{-1} is a symmetric stretch of the COO from the carboxylate groups. The band at 1030 cm^{-1} is an antisymmetric stretch (C-O-C) given by the guluronics units (Sartori et al., 1997).

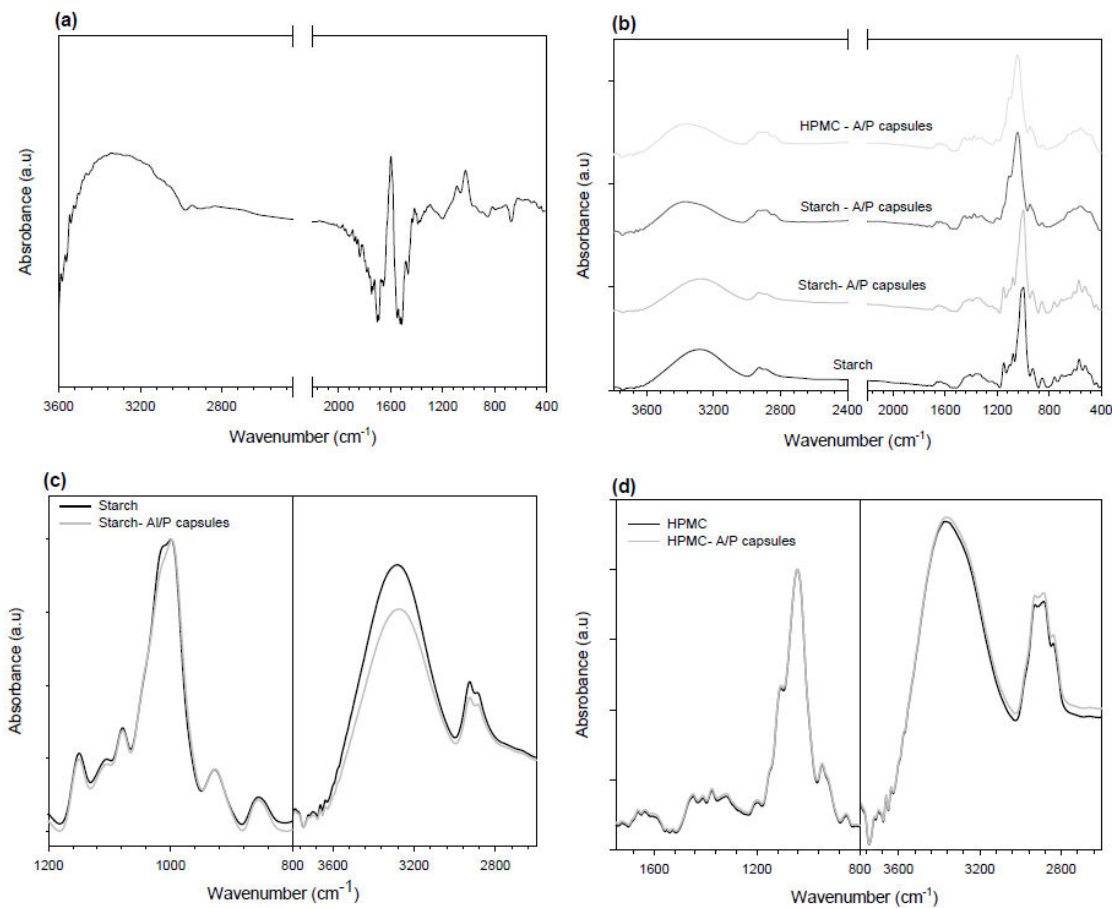


Figure 35. FTIR spectra of A/P microbeads (a), starch and HPMC films with and without A/P microbeads incorporation (b), magnification of starch films (c) and HPMC films spectra (d).

The region between 1000 and 1140 cm^{-1} corresponds to the stretching vibrations of (C–OH) side groups and the (C–O–C) glycosidic bond vibration from pectin molecules. Finally, the

absorption bands between 1100 and 1200 cm^{-1} were from ether (R–O–R) and cyclic C–C bonds in the ring structure of pectin molecules (Synytsya et al., 2003).

The FTIR spectra of the preconditioned films in the presence and absence of A/P microbeads are shown in Fig. 2b. In general, films with and without A/P microbeads present similar features in the FTIR spectral regions.

Starch film spectra showed characteristic bands at 931 and 1149 cm^{-1} , which are associated with the C-O bond stretching. The peaks at 1016 and 1077 cm^{-1} are characteristic of the C-O stretching of the anhydroglucose ring and the peak at 1645 cm^{-1} is related to O-H stretching of water molecules linked to starch structure.

Finally, the band between 3100 and 3600 cm^{-1} due to (O-H) stretching is followed a band at 2929 cm^{-1} is associated with the C-H stretching in the glucose ring.

The region below 800 cm^{-1} displayed complex vibrational modes due to the skeletal vibrations of the pyranose ring in the glucose unit (Kizil et al., 2002).

The incorporation of A/P microbeads in the starch film results in very little modifications. In fact, the absorption band at 3300 cm^{-1} showed a decreased intensity (Fig. 2c), and the band around 1000 cm^{-1} showed a narrow width in the presence of A/P microbeads. These spectra modification could be related to potential interaction between alginate (COO^-) and starch (–OH) groups, via hydrogen bonding (Swamy et al., 2008).

Although, the FTIR spectra of starch before and after functionalization with A/P microbeads, show only slight differences, they were repeated in all the tests made on different samples.

The HPMC FTIR spectra with and without A/P microbeads incorporation showed the same typical band with neither significant different intensities nor evident shifts (Fig 2b and d). The band situated at 3000-3750 cm^{-1} corresponding to the hydroxyl groups stretching (O-H), is followed by a small band at 300-2800 due C-H stretching.

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The small band at 1640-1650 cm^{-1} indicated the C-O of the HPMC pyranose molecules or could be related to O-H stretching of water molecules coupled to the structure of HPMC (Klangmuang et al., 2016).

The sharp band at 1050 cm^{-1} (C-O stretching) presented an evident shoulder at 1110 cm^{-1} attributed to a C-O-C asymmetric stretching vibration (Akhtar et al., 2012).

In the same sense, previous studies of nano-functionalized films showed that incorporation of particles didn't not resulted necessarily in important FTIR spectra modifications (García et al., 2009).

2.3. Viability of lactic acid bacteria during storage of the films.

The viability of free and encapsulated *L. lactis* added to HPMC and Starch films was tested throughout a storage period of 12 days at 5°C and 75% RH. *L. lactis* microbial counts as a function of the storage time are shown in Fig.3a.

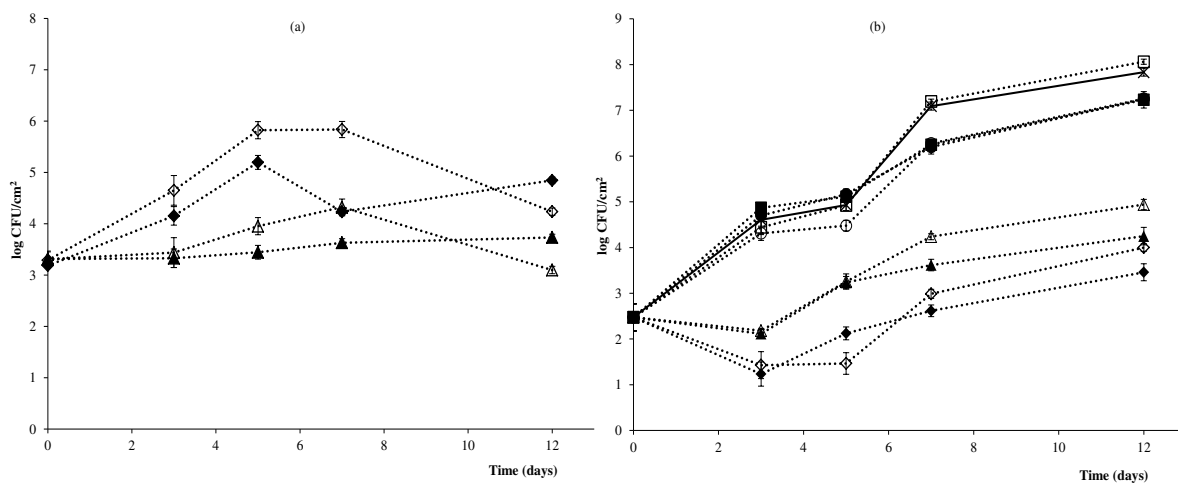


Figure 3. Effect of bioactive films on survival of *Lactococcus lactis* in the film in contact with the culture medium (a) and growth of *Listeria monocytogenes* (b) on TSA medium, stored at 5°C. Mean values and standard deviation. (□ HPMC, Δ HPMC+B, ○ HPMC+C, ◇ HPMC+C+B, ■ ST, ▲ ST+B, ● ST+C, ◆ ST+C+B and control in solid line). B= Bacteria; C= Capsules.

As can be seen, the viability of encapsulated *L.lactis* was greater than that free bacteria in both polymer matrices. For free *L.lactis*, a significant reduction of the initial population was observed during the storage period, which indicated that free *L.lactis* were more sensitive to

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storage stresses. By comparing both hydrocolloid matrices, starch appeared to be a more favorable environment for *L.lactis* survival.

Regardless of the nature of the matrix, worse results were obtained free *L.lactis* in comparison with encapsulated *L.lactis*. Counts for free *L.lactis* were lower than 2 log CFU/cm² in all films after 5 days storage, which indicates the great sensitivity of this strain to the lack of nutrients and to the decrease of the water content.

2.4. Antilisterial activity

The antimicrobial activity of the developed films against *Listeria monocytogenes* was tested in a synthetic non-selective medium (TSA) stored at 5°C (Fig. 3b). Pure HPMC and Starch films and with A/P capsules without *L. lactis* were used as control samples. As is shown in Fig.3b, *L. monocytogenes* population increased from 2.5 to 7.5 log CFU/cm² at the end of the storage period. As expected, pure HPMC and Starch films with A/P capsules without *L.lactis* were not effective against *L. monocytogenes* growth, since no significant differences were observed in microbial growth on TSA plates.

All films containing bioactive cultures exhibited a significant antilisterial activity since, during the period of storage, a reduction of the initial microbial population was observed in all cases. The films with free and encapsulated *L. lactis* showed, therefore, bactericidal activity. After 3 storage days, the best growth limitation were obtained with starch and HPMC films with encapsulated *L.lactis*, all films with free and encapsulated *L.lactis* led to a reduction of the microbial growth of approximately 3 logs with respect to the control.

For films based on polysaccharide, different results were obtained. The initial population remained constant during the first 7days and, after that, a slight decrease was observed in agreement with Gialamas et al. (2010) and Sánchez-González et al. (2014). In this sense, the

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viability of free *L. lactis* significantly decreased in comparison with encapsulated *L. lactis* and limited *L. monocytogenes* inhibition.

3. Conclusion

Encapsulation of *L. lactis* in alginate conjugated with entrapment in starch or HPMC films shown a high efficiency to produced active packaging materials. The mechanical, transport and transparency properties remained adapted to packaging application despite of capsules incorporation. Moreover, the developed films with microcapsules presented an interesting antilisterial activity. These systems must be considered as a possible solution for active packaging in the near future.

Acknowledgements

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Chapter VI: General Conclusion

Lactococcus lactis subsp. lactis est connu pour son activité antagoniste contre *Listeria Monocytogenes*. Nous avons tenté d'utiliser cette souche comme composant d'un emballage actif par microencapsulation. Le but de cette étude était en effet de développer de nouvelles microsphères d'hydrogel (alginate-pectine) pour la microencapsulation de *L. Lactis*. Deux principaux types d'encapsulation ont été distingués, l'un correspondant à un « type matrice » (ou microbilles) et l'autre « type coeur – couronne » (ou capsules).

Les microsphères contenant les bactéries lactiques ont été préparées par extrusion. Le ratio des deux biopolymères utilisés, alginate/pectine (A/P) a varié comme suit: 100/0, 75/25, 50/50, 25/75, 0/100. Différents milieux permettant la survie de *L. lactis* ont été utilisés (milieu de culture M17 enrichi en glucose ou l'eau physiologique) pour contenir les bactéries à incorporer dans deux états physiologiques différents (phase exponentielle, phase stationnaire).

Les résultats ont permis de déterminer le ratio « A/P » le plus efficace pour augmenter les propriétés des billes et la survie des bactéries. Les meilleures propriétés mécaniques ont été obtenues pour le ratio 75/25 ; Les billes se sont montrées plus stables et ont permis une meilleure libération de nisine au cours de la période de stockage de 7 jours à 30 °C.

L'état physiologique des bactéries au cours du procédé d'encapsulation ainsi que le milieu d'encapsulation ont été démontrés comme deux facteurs déterminant de la viabilité et l'activité de production de bactériocine de *L. lactis*. Les meilleurs résultats ont été obtenus avec des souches encapsulées en phase exponentielle de croissance dans un milieu M17 additionné de 0,5% de glucose.

Dans un deuxième temps, des microcapsules ont été préparées par la formation d'une membrane d'hydrogel d'alginate et d'un cœur liquide, composée de M17 complété de 0,5% de glucose ou d'eau physiologique enrichi en gomme xanthane pour en modifier la viscosité. Le but de

cette étude était de développer des microcapsules à cœur aqueux, pour analyser les propriétés physico-chimiques de ces microcapsules, d'évaluer leur impact sur la survie des bactéries encapsulées en phase exponentielle ou stationnaire. De plus, la libération et l'activité antilisterial de nisine produite ont été suivies sur 10 jours. Les résultats ont montré que la composition du cœur liquide des microcapsules n'a pas affecté la stabilité du réseau d'alginate et que les capsules ont bien résistées aux conditions de conservation. L'addition de gomme xanthane dans le cœur a permis, dans un premier temps, de contrôler la viscosité et une bonne formation du cœur sphérique dans le centre des microcapsules; mais a aussi renforcé la structure des capsules par établissement de liaison hydrogène entre la gomme xanthane et les groupes d'hydroxyles de l'alginate.

Les microcapsules de *L. lactis* encapsulé à phase exponentielle dans une membrane d'alginate et dans un cœur à base de M17 enrichi avec 0,5% de glucose et 0.2% de gomme de xanthane ont donné les meilleurs résultats.

Ces capsules ont enfin été incorporées dans des films d'Amidon et d'HPMC et un excellent effet antilisteria a été démontré ce qui est prométeur pour le la préparation d'emballages actifs.

Les films enrichis en microcapsules après quelques études de transfert de technologie vers l'industrie pourront être finalisés pour la conservation des aliments hudides à courte durée de vie. Ces nouveaux films bioactifs, à base de biopolymères (amidon, HPMC) et enrichis avec *L.lactis*, incorporés et stabilisés dans un milieu composé d'alginate/gomme de xanthane à cœur et d'alginate /pectine pour la membrane pourront alors être utilisés.

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Résumé

La nisine est un peptide antimicrobien produit par la souche de *Lactococcus lactis subsp. lactis*, autorisée pour des applications alimentaires par le comité d'experts sur les additifs alimentaires et les aliments de l'Organisation des Nations Unies pour l'Alimentation et l'Agriculture et de l'Organisation Mondiale de la Santé (FAO / OMS). La nisine peut être appliquée, par exemple, pour la conservation des aliments, la biopréservation, le contrôle de la flore de fermentation, et potentiellement comme agent antimicrobien clinique. Le piégeage de bactéries capables de produire de la nisine dans des billes d'alginate de calcium est ainsi une voie prometteuse pour immobiliser des cellules actives et prolonger la durée de conservation des aliments.

Le travail de thèse visait à concevoir des emballages actifs en biopolymère renfermant des bactéries lactiques bioprotectrices (LAB) pour contrôler la croissance de micro-organismes indésirables dans les aliments, en particulier *L. monocytogenes*. La stabilité mécanique et chimique des billes d'alginate a d'abord été améliorée, et l'efficacité d'encapsulation a été accrue. Des capsules « alginate / pectine (A / P) » ont été préparées comme premières microsphères, par une technique d'extrusion. La production de nisine par *Lactococcus lactis subsp. lactis* encapsulé dans différents états physiologiques (phase exponentielle, phase stationnaire) a été étudiée. Les résultats ont montré que les billes composites (A/P) avaient de meilleures propriétés que celles formulées avec de l'alginate ou de la pectine pure. L'association de l'alginate et de la pectine induit un effet synergique qui a amélioré les propriétés mécaniques des microbilles. La deuxième partie du travail a concerné la mise au point de microcapsules à cœur liquides avec une membrane d'hydrogel d'alginate et d'un noyau de gomme de xanthane. Les résultats ont montré que ces microcapsules contenant *L. lactis* encapsulé durant la phase exponentielle dans une matrice d'alginate et un noyau nutritif de gomme xanthane ont donné les meilleurs résultats et présentent une activité anti-listeria intéressante. Ces microbilles ont enfin été appliquées pour la conservation des aliments et en particulier dans des emballages alimentaires actifs. Des films (HPMC, amidon) ont été élaborés en piégeant des perles d'«alginate actifs/gomme xanthane» enrichies en *L. lactis* dans des films d'emballage et appliqués pour la conservation des aliments.

Abstract

Nisin is an antimicrobial peptide produced by strains of *Lactococcus lactis subsp. lactis*, recognized as safe for food applications by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO). Nisin could be applied, for shelf-life extension, biopreservation, control of fermentation flora, and potentially as clinical antimicrobials. Entrapment of bacteria able to produce nisin in calcium alginate beads is promising way for cells immobilization in active films to extend food shelf-life.

The present PhD work aimed to design biopolymeric active packaging entrapping bioprotective lactic acid bacteria (LAB) and control undesirable microorganisms growth in foods, particularly *L. monocytogenes*. First, the mechanical and chemical stability of the alginate beads were improved, and consequently the effectiveness of encapsulation was increased. Alginate/pectin (A/P) biopolymers were prepared, as first microspheres design, by extrusion technique to encapsulate nisin-producing *Lactococcus lactis subsp. lactis* in different physiological state (exponential phase, stationary phase). Results showed that A/P composite beads were more efficient to increase beads properties than those formulated with pure alginate or pectin. Association of alginate and pectin induced a synergistic effect which improved microbeads mechanical properties. As a second microspheres design, aqueous-core microcapsules were prepared with an alginate hydrogel membrane and a xanthan gum core. Results showed that microcapsules with *L.lactis* in exponential state encapsulated in alginate membrane and aqueous-core based on xanthan gum with nutrients gave the best results and exhibit interesting antilisterial activity. These microparticles were applied in food preservation and particularly in active food packaging. A novel bioactive films (HPMC, starch) was developed and tested, entrapping active beads of alginate/xanthan gum core-shell microcapsules and alginate/pectin hydrogel enriched with *L.lactis*.