

**EFFECT OF MICROENCAPSULATION
ON FUNCTIONAL AND TECHNOLOGICAL
FEATURES OF PROBIOTIC AND STARTER
CULTURES**

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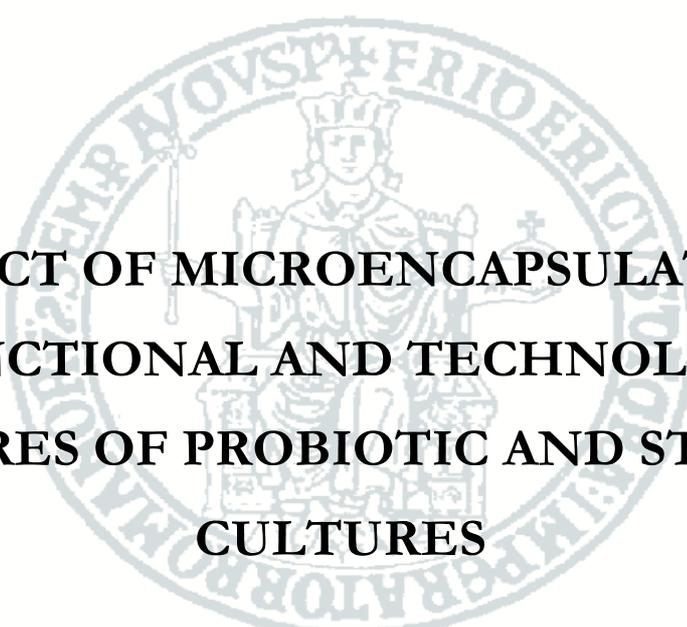
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**EFFECT OF MICROENCAPSULATION
ON FUNCTIONAL AND TECHNOLOGICAL
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CULTURES**

Annachiara De Prisco

Thesis

submitted in fulfilment of the requirements for the degree of doctor
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LIST OF ABBREVIATIONS

GI; gastrointestinal

LAB; Lactic Acid Bacteria

AA; alginate matrix microcapsules

AM; chitosan coated alginate matrix microcapsules

FAA; freeze-dried alginate matrix microcapsules

FAA; freeze-dried chitosan coated alginate matrix microcapsules

EE; entrapment efficiency

XM; chitosan coated alginate-xanthan matrix microcapsules

CAM; chitosan coated alginate core-shell microcapsules

CXM; chitosan coated alginate-xanthan core-shell microcapsules

SSS; simulated saliva solution

GSS; simulated gastric solution

ISS; simulated intestinal solution

CHAPTER 1

Brief thesis presentation

1.1 Short overview

Microencapsulation is currently described as the technique that permits the physical containment into functional matrices of several categories of compounds that can benefit from this procedure. Vitamins, polyphenols, bacteriocins, living cells and other many sensitive compounds can be efficiently protected, delivered, released and easier handled when in microencapsulated form during their utilization and storage. Microencapsulation of living cells has gradually become an important area of intense research and industrial development within the wider field of microencapsulation. First applications of encapsulated microorganisms in the food sector were mainly confined to fermentation processes in which a higher recovery of enzymes, ethanol, lactic acid or antimicrobial peptides alongside a higher cell density were obtained with the use of encapsulated microorganisms. More recently, the encapsulation of probiotic bacteria has gained a great attention because of the undoubted potentiality of this technology to provide protection toward probiotics by enhancing their capability to cope with different stress factors. Probiotics are defined as live microorganisms able to confer health benefits to the host when administered in adequate amounts and, in order for probiotics to preserve their expected health-promoting effects, they need to survive during passage through the gastrointestinal tract. Accordingly, the most investigated area in the field of probiotic encapsulation is the one dealing with the development of microcapsule systems able to protect bacteria against gastrointestinal barriers and to efficiently mediate their delivery to the intestine, their target site of action. Furthermore, because probiotics are becoming an important resource for the maintenance and restoration of the normal intestinal microbiota, and in general for the human wellbeing, a great attention is currently given to the extension of the categories of foods carrying probiotics. At this regard, microencapsulation is also regarded as a valid strategy for the improvement of probiotic viability against technological and food-related hurdles in order to i) increase their survival during the manufacturing and the storage of foods and iii) to contribute to the development of innovative probiotic food carriers. Despite the relevant improvements toward the useful application of microencapsulation technology to probiotic microorganisms, some challenges still exist and need to be addressed to enable wider industrial acceptance of cell microcapsules in various production processes. Typical examples are the development of easy to use, stable and cheap microcapsules suitable for food applications, the monitoring of cell stability along the entire food production including a real storage period and the assessment of the effect of new micro-environment created within the capsules on cell metabolism.

This PhD thesis deals with this context.

1.2 Aims and outline

The overall objective of this research is to contribute to the evidences supporting the application of microencapsulation technology for the improvement of living cell resistance and functionality. This general aim has been addressed through the accomplishment of three main research topics such as i) the improvement of probiotic cell survival and functional features under stress conditions; ii) the evaluation of the effect of microcapsules on technological and functional properties of starter culture and iii) the investigation at cellular level, through application of proteomics, to unravel fundamentals supporting increased cell viability attributed microencapsulation. These objectives were achieved making use of a research plan structured according to the following thesis outline.

The first step toward the achievement of the aim of this thesis was to validate the extrusion vibrational nozzle technology, for the first time used in our laboratories, at the encapsulation of living cells. Hence, the *Chapter 3* reports the production of alginate and chitosan coated alginate-based microcapsules loaded with probiotic *Lactobacillus reuteri* DSM 17938 cells according to the matrix microcapsule morphology (see pf. 2.2 of the Literature Review at Chapter 2). This encapsulating system has been tested for entrapment efficiency and cell viability, for capability to produce microcapsules with suitable morphological properties for food application and for the attitude of the resulting microcapsules to resist to lyophilisation process and to protect probiotic cells during some preliminary tests (e.g. gastrointestinal passage).

Chapter 4 has as main topic the tailoring of chitosan coated alginate matrix microcapsules. Indeed, this base microcapsule type has been modified in its composition, for the addition of xanthan gum used as filling agent and in its structure, for the development of core-shell type microcapsules (see pf. 2.2 of the Literature Review at Chapter 2). This Chapter also starts the experiments aimed at the determination of the best microcapsule systems in terms of cell protection. At this purpose, all microcapsule systems produced have been further investigated for their ability to protect probiotic cells under different thermal stress conditions.

Same microcapsule systems have been applied in experiments reported in *Chapter 5* for the assessment of their ability to protect new isolated, interesting and potentially probiotic lactobacilli that exhibited poor native ability to withstand the stress for gastrointestinal environment. These experiments have been performed according a more complex protocol for a better simulation of the human gastrointestinal conditions than the one followed in Chapter 1, in order to study the effect of the new solutions (saline based solutions plus lysozyme or digestive enzymes and bile salts) on microcapsules and on strain viability.

Results produced and reported in Chapters 3, 4 and 5 allowed the utilization of the most suitable microcapsule types for their application in real food systems. Accordingly, *Chapter 6* focused on the application of microencapsulated probiotics in three different food matrices selected on the base of: i) differences in the nature of stress factors they pose against probiotic viability and ii) broadening the categories of food products functionalized and delivering probiotics.

Chapter 7 arises from the need to understand the effect of microcapsules here applied on metabolic features of bacteria. At this purpose, a yoghurt starter culture has been

microencapsulated and its ability to ferment milk has been evaluated alongside the production of volatile compounds through GC-MS to assess the effect of new microenvironment created by the capsules onto microbial activity. Furthermore, by using a commercial probiotic *Lactobacillus delbrueckii* as component of the yoghurt starter culture, it was also possible during the same experiments to test the aptitude of alginate-based microcapsules to protect bacteria along the production, the storage and simulated digestion of a food.

Chapter 8 reports on a preliminary investigation through proteomic approach about the possible cellular reasons that can account for the increased resistance of encapsulated probiotic bacteria during simulated gastrointestinal transit. These experiments also aim at the validation of proteomics by 2D SDS-PAGE coupled to MALDI TOF as useful tool for the investigation of possible molecular modifications of encapsulated bacteria under several stress conditions or during the many different processes carried out with microencapsulated bacteria.

The last chapter, the *Chapter 9*, presents a critical synthesis of the main findings achieved from the entire research, and conclusions and recommendations based on knowledge generated.

CHAPTER 2

Literature Review

2.1 General introduction

Cell microencapsulation has its roots in the primordial microbial immobilization technique, referred to as the procedure for physical confinement of cells to a certain defined space with the main purpose of preserving their viability. Through the tailoring of innovative techniques and the research on new suitable encapsulating materials, microencapsulation has become a sophisticated technology for the entrapment of living cells targeting a variety of applications. It can be defined as the engineered inclusion of cells into functional matrices to form solid objects with variable size (1-1000 μm), shape (e.g. spherical, droplet, irregular) and morphology (e.g. coated/non-coated matrix or reservoir microcapsules, single/multi-core, single/multi-layers). Currently, cell microencapsulation still accounts many applications in the research field, as suggested by the continuous growth in scientific publications, along with the scaling-up of the technology for the industrial development. Initial research on living cell immobilization dates back to 70-80's. Typical initial research efforts include the demonstration that this procedure enhanced aspartase activity in *E. coli* (Chibata et al., 1974); that when yoghurt bacteria (*L. delbrueckii* and *S. thermophilus*) were entrapped in different alginate particles with the aim to improve a continuous pre-fermentation of milk for yoghurt production (Prévost and Diviès, 1987; 1988a and b). The use of immobilized microbial cells in several biotechnological processes was found to be more profitable over the utilization of same cells in free form (Rathore et al., 2013). This practice was found advantageous in a number of industrial applications including improved production of some metabolites (e.g. lactate, ethanol), facilitating the separation of cells from fermentation products, enhancing fermentation process due to higher cell density achieved, permitting the reuse of immobilized cells. In addition, the procedure has also been proved to enhance cell endurance against environment stress-related factors and thus prolonging cell viability. Because of the versatility of immobilization, and later of the microencapsulation, for the entrapment of many different microbial cells, this technology is still being explored in as many different applications. Typical examples, including the production of biofuels, environmental decontamination, novel food development and enzymes, vitamins, food and pharmaceutical products are presented in table 2.1. In the field of food science and technology, immobilization/microencapsulation technology gradually became a tool for the improvement of the performances of microorganisms in various areas of technological interest (e.g. starter cultures, biocatalysts). In addition, the technology is also being applied as a strategy for protection of microorganisms considered beneficial (i.e. probiotics) to human and/or animal health.

Table 2.1

Applications and purposes of immobilized/encapsulated microbial cells in different technological processes

Encapsulated cells	Immobilization/encapsulation carrier	Purposes of application	Reference
• Biofuel production			
<i>Clostridium acetobutylicum</i> SE25	AquaMats AO	Enhancement of butanol production	Li et al., 2016
<i>Synechocystis</i> sp. PCC6803	HFE-7500 oil	Increment of cell cyanobacteria photosynthetic growth and production of lactate	Hammar et al., 2015
• Environmental decontamination			
<i>Anthracoxyllum discolor</i> Sp4 CCCCT 16.5	Alginate-CaCl ₂	Degradation of herbicide atrazine	Elgueta et al., 2016
<i>Ogataea polymorpha</i> VKMY-2559	Organic silica sol-gel	Purification of methanol containing industrial wastewater	Kamanina et al., 2016
<i>Lysinibacillus fusiformis</i> B26	Agar and calcium alginate	Decolourization of synthetic dye	Dogan et al., 2016
• Production of ingredients for food industry			
<i>Lactococcus lactis</i> ; <i>Lactobacillus plantarum</i> NCIM 2084; <i>Lactobacillus delbrueckii</i> NCIM 2365;	Hexamethylene diisocyanate and glutaraldehyde and chitosan; Polypropylene matrix and chitosan; Palmitoylated alginate	Enhancement of lactic acid production	Groboillot et al., 1993; Krishnan et al., 2001; Rao et al., 2008
<i>Saccharomyces cerevisiae</i> , mixed starter culture from Loog-Pang (Thai rice cake)	Carboxymethylcellulose-alginate- CaCl ₂ ; Silk cocoons	Enhancement of ethanol production	Talebnia and Taharzadeh, 2007; Khamkeaw and Phisalaphong, 2016
<i>Candida guilliermondii</i>	Alginate- CaCl ₂	Xylitol production	Carvalho et al., 2003
<i>Enterococcus faecium</i> A2000; <i>Lactobacillus curvatus</i> MBSa2	Alginate-CaCl ₂	Enhancement of bacteriocin production	Ivanova et al., 2000; Barbosa et al., 2015
<i>Streptomyces</i> sp. RCK-SC	Polyurethane foam (PUF)	Enhancement of pectinase production	Kuhad et al., 2004
• Fermentation of food matrices			
Lactoferm ABY 6;	Chitosan coated alginate-CaCl ₂	Fermentation of whey based substrate and improvement of bacterial survival	Krunić et al., 2015
<i>Lactobacillus plantarum</i> PCS 26	Alginate-CaCl ₂	Fermentation of apple juice and prolongation of probiotic viability	Dimitrovski et al., 2015
<i>Lactobacillus plantarum</i> and <i>Staphylococcus xyloso</i>	Alginate-starch	Improvement of cells viability during meat thermal treatment for fermented sukuc preparation	Bilenler et al., 2017
Probiotic <i>Lactobacillus delbrueckii</i> and <i>Streptococcus thermophilus</i>	Chitosan coated alginate-CaCl ₂	Elucidation of fermentative ability of encapsulated starter cultures and protection of probiotic strain	De Prisco et al., in submission

Table continues in the next page

• <i>Protection of probiotics and enhancement of their functions</i>			
<i>Lactobacillus reuteri</i> CGMCC 1.3264	Fe ₃ O ₄ particles	Enhancement of reuterin production	Lui and Yu, 2015
<i>Lactobacillus reuteri</i> DSM17938	Alginate-CaCl ₂	Improvement of cell viability during storage	De Prisco et al., 2015
<i>Lactobacillus gasseri</i> and <i>Bifidobacterium bifidum</i> ; <i>Lactobacillus plantarum</i> TN8	Chitosan-alginate-CaCl ₂	Improvement of cell viability during GI simulated transit	Chávarri et al., 2010; Trabelsi et al., 2013
• Development of new foods			
<i>Lactobacillus rhamnosus</i> GG; <i>Bifidobacterium animalis</i> BB-12	Whey proteins; cellulose acetate phthalate	Probiotic fruit juices	Doherty et al. 2012; Antunes et al., 2013 al.,
<i>Lactobacillus reuteri</i> DSM 17938	Chitosan coated alginate-CaCl ₂	Probiotic chocolate soufflé	Malmo et al., 2013
<i>Lactobacillus casei</i> ATCC 39392	Calcium alginate-resistant starch	Probiotic cream filled cake	Zanjani et al., 2012

2.2 Generalities on microencapsulation technology

As previously mentioned, microencapsulation technology evolved from the principle of cell immobilization. In broader sense, microencapsulation can be defined as the technology of holding sensitive compounds in/onto functional matrices as illustrated in Fig. 2.1. This research focused mainly on the aspects of entrapment and containment procedures. In the practice, microencapsulation step includes the complete envelopment of pre-selected core materials in either liquid, solid or gaseous phase within a defined natural or synthetic porous or impermeable membrane by using different techniques (De Vos et al., 2010; Whelehan and Marison 2011).

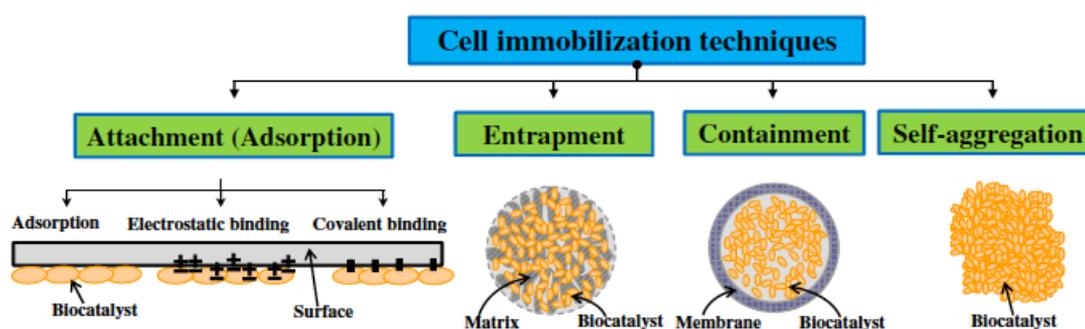


Fig. 2.1 Types of cell immobilization techniques. Image by *Abbel-Rahman et al., 2013*

The products that comes from microencapsulation procedure are the microcapsules, solid particles that can differ for their size (Fig. 2.2) and morphology (Fig. 2.3) depending on materials and techniques applied to produce them. Capsule size varies and range from macro (>1000 μm), micro (1-1000 μm) or nano (<1 μm) ranges. Encapsulation technology applied to microbial cells, which are typically 1-4 μm in size, is usually referred to as microencapsulation (Gawkowski and Chikindas 2013). According to their morphology, microcapsules can be classified in five main different types (Gharsallaoui et al. 2007; Solanki et al., 2013) as illustrated in Fig. 2.3. In the “core-shell” or “reservoir” microcapsule type, cells or other sensitive compounds are retained in the core of the capsules completely surrounded by a continuous entrapping membrane. Both the diameter of the core and the thickness of the membrane can vary. In the case of the “multicore” system the encapsulated ingredients form

two or more aggregations, as usually occurred in emulsification techniques (Whelehan and Marison 2011). When capsules are not characterized by a well-defined and spherical morphology they are defined “irregular” shaped.

Emulsification								} Matrix type
Extrusion								
Spray drying								} Reservoir type
Spray coating								
Coextrusion								
0.01 0.1 1 10 100 1000 10000								
Size range (μm)								

Fig. 2.2 Capsule size ranges for main techniques used in cell encapsulation for food and pharmaceutical application. Image by Solanki *et al.*, 2013.

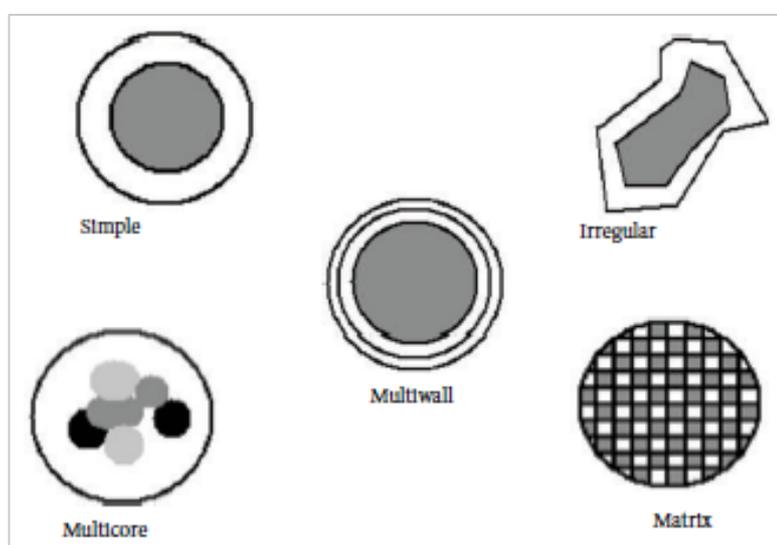


Fig. 2.3 Different types of microcapsules used in food industry. Image by Solanki *et al.*, 2013.

In “matrix” type microcapsules, a physical separation of the encapsulating agent and the encapsulated ingredient does not exist since the latter is homogeneously dispersed to create a network with the encapsulating agent so that it is present in the entire microcapsule area. All microcapsule types reported above can be coated by applying a further layer of a coating agent that can create bonds with capsule material due to physical or chemical reactions; resulting particles can be referred as “multiwall” microcapsules. Besides capsule sizing, microcapsules morphology is an important parameter to be tailored during cell encapsulation, since it is reasonable a correlation between microcapsule morphology and their performances.

The different types of microcapsules are produced from a wide range of wall materials and by a large number of different microencapsulation processes including spray-drying, spray-cooling, spray-chilling, air suspension coating, extrusion, centrifugal extrusion, freeze-drying, coacervation, co-crystallization, liposome entrapment, interfacial polymerization, emulsion or micro-channel and membrane emulsification. (Anal & Singh, 2007; Gharsallaoui *et al.*, 2007;

De Vos et al., 2010). Among these, extrusion, emulsion and spray drying techniques in particular are the most suitable to be scaled-up for industrial production of cell microcapsules (Burgain et al., 2011). Many comprehensive reviews (Anal & Singh, 2007; De Vos et al., 2010; Rokka and Rantamäki, 2010; Corona-Hernandez et al., 2013; Martín et al., 2015) have already dealt with major aspects of microencapsulation techniques, including methodologies and materials used for cell encapsulation. Therefore, this section of the thesis will only provide the most relevant information about materials (alginate, chitosan and xanthan gum) and technique (vibrational nozzle technology) selected for the present research.

2.2.1 Encapsulation techniques: a focus on vibrational nozzle technology

Although it is impossible to declare the most suitable technology in the field of cell encapsulation, spray drying, emulsion and extrusion are the most common microencapsulation techniques applied to living cells, despite many inherent disadvantages. Spray drying is the most widely utilized technique and it is cheap and easy to be scaled-up; however, it entails the application of high temperature that very often lead to the damage of cells (Anal and Singh 2007), with the consequent loss of bacteria viability and resistance during their further utilization. Even though emulsion is a simply and easy to scale-up technique and it does not induce cell mortality, it is an expensive method that produces varying size and shape of microcapsules (Burgain et al. 2011). Furthermore, it requires additional steps to remove residues of oil used during the process. With respect to extrusion, it is a simple and cost-effective way to manufacture homogeneous-shaped capsules with a narrow size distribution, which could be designed for mass production of microcapsules; furthermore new systems based on extrusion principles to adapt microcapsule production to industrial scale are being developed (Burgain et al., 2011; Whelehan and Marison 2011).

Microencapsulation via extrusion technique is based on the principle of generating droplets from the extrusion of polymer matrix used as encapsulating agent, previously mixed with the ingredient to be encapsulated (here called cell-polymer suspension), through a nozzle of different diameter. By coupling this base-process of extrusion to mechanical means such as vibration forces it is possible to increase and tailor the normal dripping process at the orifice. The technology based on extrusion and vibration forces is the vibrational nozzle technology or vibrating technology and its potential use in living cell encapsulation has been considerably investigated (Brandenberger and Widmer., 1998; Heinzen et al., 2004; Mazzitelli et al., 2008). In the vibrating technology controlled application of vibrational/oscillation frequency causes the break of the laminar jet produced when the cell-polymer suspension is extruded through a nozzle at certain flow rates (Whelehan and Marison 2011). Due to the action of surface tension forces, broken segments from the flow stream form spherical droplets. The characteristics of the droplets depend on the nozzle diameter, the flow rate, the intensity of vibration frequency (at defined amplitude), viscosity of the liquid extruded (Serp et al. 2000) and the distance between the outlet and the polymerizing solution (Anal and Stevens 2005). By applying a permanent sinusoidal force at a defined frequency level to the cell-polymer suspension jet stream, the break-up of laminar jets can be controlled to form one droplet with uniform, equal size per hertz of applied frequency (Rayleigh 1879) as depicted in Fig. 2.4. Vibrating technology has been recently reported as a suitable technology for the production of mono-dispersed, uniform in shape and size microcapsules for food and pharmaceutical application using a short time for capsule production (Whelehan and Marison 2011).

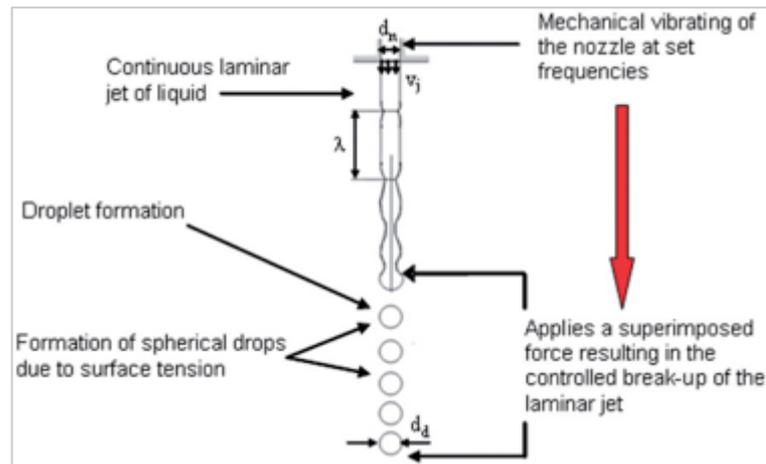


Fig. 2.4 Controlled break-up of a laminar liquid jet into droplets of equal size. (*Heinzen et al. 2004; Whelehan and Marison 2011*).

Since the use of solvent or other chemicals is not required, it is particularly suggested for cell encapsulation (Graaf et al., 2008; De Prisco et al., 2015) as well as for other food ingredients such as polyphenols and, in our laboratory, bacteriocins (Aizpurua-Olaizola et al., 2016; Maresca et al., 2016). Recently, a new device, the Encapsulator B-395 PRO, implementing the vibrational nozzle technology has been developed by BÜCHI (BÜCHI Labortechnik, Flawil, Switzerland). The device is additionally equipped with an electrostatic dispersion unit, designed to negatively charge the droplet surface immediately after their extrusion. The repulsion forces generated among the droplets due to the equally charged surface let the particles to repel each other, dropping separately in the polymerising bath. The electrostatic charge generator avoids the formation of capsule coalescence and permit the recovery of microcapsule population free from agglomerates. Schematic illustration of cell encapsulation according to the vibrational nozzle technology is presented in Fig. 2.6. Living cells or other ingredients to be encapsulated are mixed with the polymer selected as encapsulating agent (1) before being feed to the instrument by the delivery apparatus i.e. precision syringe pump (2) or a pressure bottle system. Feeding rate, measured in ml/min, can be regulated by the “pump” function for the syringe pump system (0-60 ml/min), while when the pressure bottle system is used as feeding system the feeding rate is calculated as function of the pressure applied to force the cell-suspension (0.1-1 bar). To break down the cell-suspension liquid jet into droplets of equal size, the polymer passes through a pulsation chamber (3) before reaching the nozzle. The intensity of membrane vibration can be regulated with the “frequency” function, measured in Hz (0-6000 Hz). According to the supplier instruction, number of vibration per second leads to the formation of as many particles per sec. Droplets are further extruded thanks to the passage to a nozzle (4) with a different diameter (80 to 1000 μm). Alginate solution concentration up 2.0% can be used for producing alginate microspheres. Final bead diameter is approximately twice of the nozzle diameter but bead dimensions may vary within a certain range by controlling frequency of the vibration, the feeding rate of the cell suspension and finally the intensity of electrostatic forces imposed,

allowing the production of smaller capsules as mentioned before (Serp et al. 2000; Heinzen et al. 2004; Whelehan and Marison 2011).

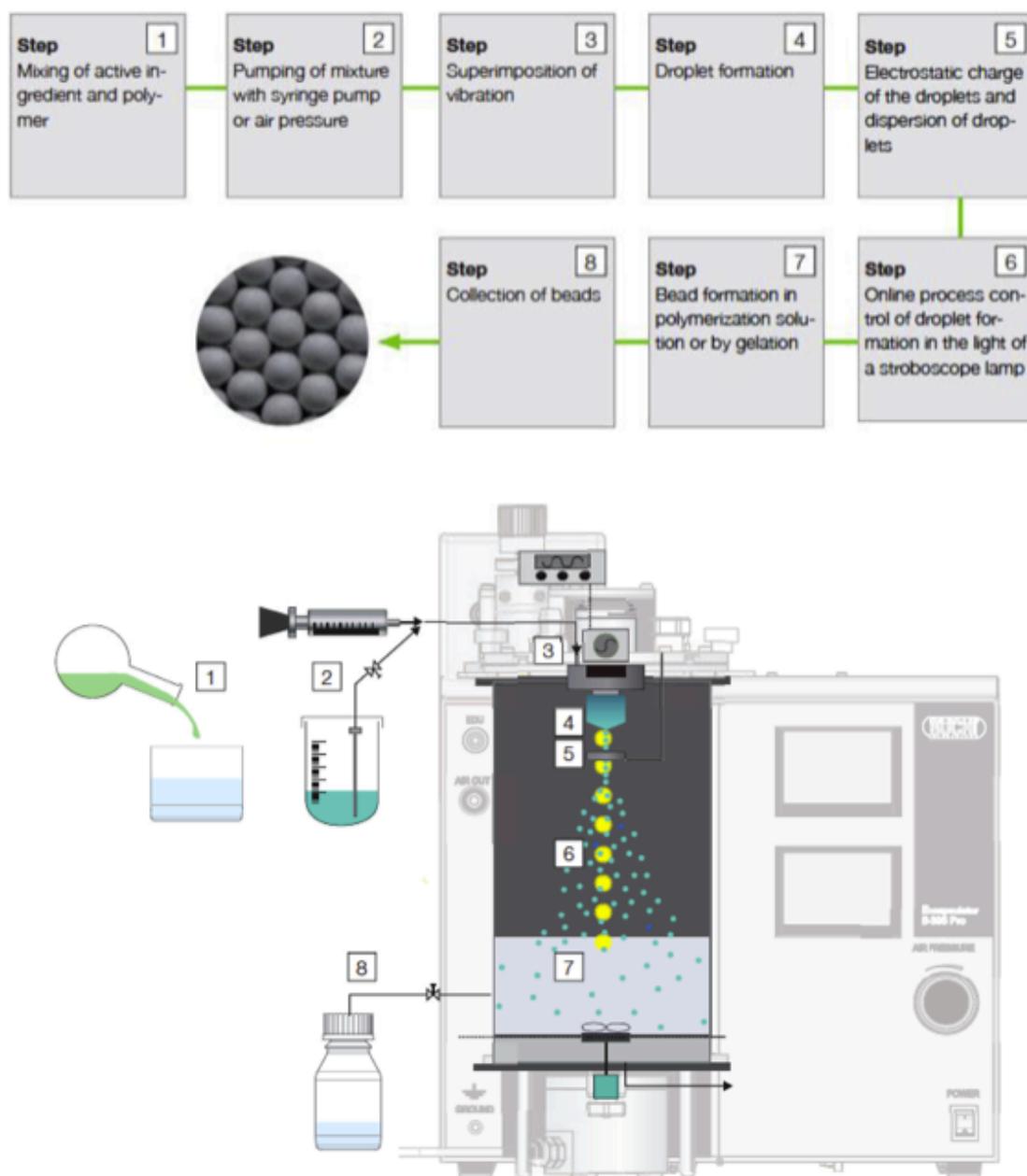


Fig 2.6 Functional principle of vibrating technology using Buchi Encapsulator B-395 Pro (BUCHI; Switzerland; De Prisco et al., 2015).

Optimal parameters of processing depends mainly on the chemico-physical characteristics of the substances used. A strong negative charge introduced by an electrode placed directly beneath the nozzle (5) causes the droplets to repel each other, resulting in the opening of the linear liquid jet into a cone-like shape (6), preventing the possible coalescence of the droplets. The magnitude of the charge necessary for satisfactory separation of the chain is heavily reliant on jet velocity, droplet diameter and concentration of the polymer solution used (Whelehan and Marison 2011). It is controlled by the “electrode” function and can be set between 0 and 2500V. Droplets are further let to harden in a polymerizing solution (7), usually

CaCl₂ solution for alginate beads, to be finally recovered by a draining system (8). What above reported can also serve as a scheme for the production of alginate matrix microcapsules. Another important element of the Encapsulator device is a concentric nozzles system which makes the instrument suitable for production of core-shell microcapsules. The inner part of the capsule, the core, can be extruded through a set of nozzles with different diameters within a range of 80-1000 μm. The outer nozzle serves for the extrusion of shell, usually made of a polymer agent such as alginate. For this purpose, a set of nozzles with diameter ranging between 200 and 1000 μm can be used. Both matrix and core-shell microcapsules can be coated with an additional layer (e.g. alginate, chitosan or poly-L-lysine, Fig. 2.7) to improve microcapsule mechanical strength and limit the diffusion of the encapsulated agents towards the outer environment and the diffusion of external compounds into the capsules.

2.2.2 Microencapsulating and coating agents: alginate and chitosan

Despite the wide array of encapsulation techniques and materials available, living cells require the most gentle technology such as extrusion in combination with suitable encapsulation agent that might preserve the functionality or even promote the functionalities for probiotics (de Vos et al. 2010). Among all materials, the most widely used for manufacture of microspheres and microcapsules for food applications include natural materials such as proteins (whey proteins), carbohydrates (starch and maltodextrin), lipids (hydrogenated fat), acacia (Arabic) gums, cellulose or synthetic polymers (Nedovic et al., 2011; Whelehan & Marison, 2011).

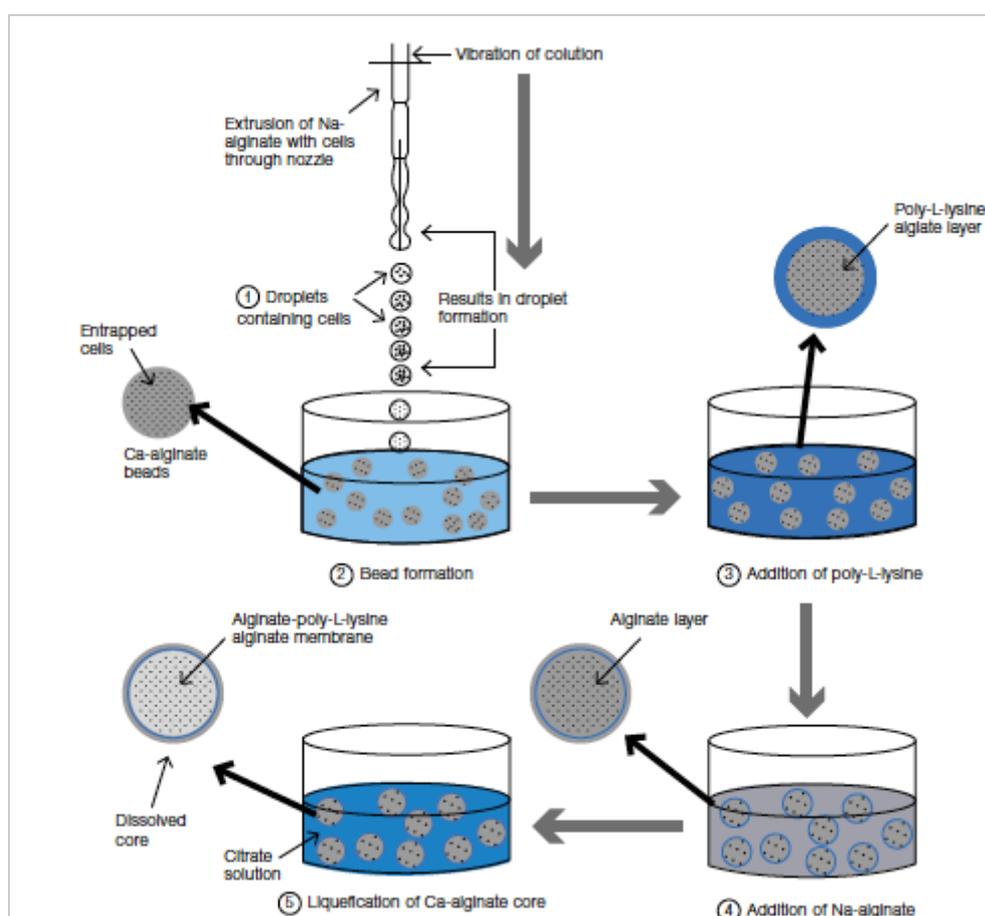


Fig 2.7 Steps for alginate cell-microcapsules multi-coating with poly-L-lysine and alginate. Similar procedure can be followed with other coating agent such as chitosan (BUCHI, Switzerland).

Moreover, the most widely used matrices used alone or in combination for encapsulation of living cells are alginate, chitosan, poly-L-lysine, whey protein, pectin, xanthan gum, carrageenan, carboxymethyl cellulose, gelatin, locust bean gum, resistant starch and gellan gum (Gbassi et al. 2011; Rodrigues et al. 2011; Rokka and Rantamäki 2015).

The vibrating technology has mainly focused on using alginate as the encapsulation matrix for several applications (biotechnological and medical), but some other polymers such as gelatin and cellulose sulphate have also been applied.

Alginate is a water-soluble gelling polysaccharides recognised as safe (GRAS) for human consumption by the Food and Drug Administration, FDA (George and Abraham 2006). It is found in great abundance as part of the cell wall and intracellular material of brown seaweeds (Phaeophyceae). Alginate is also produced by two families of heterotrophic bacteria (*Pseudomonadaceae* and *Azotobacteriaceae*) and its extraction is carried out often under strict regulatory control (Yian Wong et al., 2000). It is composed of alternating blocks of 1–4 linked α -L-guluronic (G-blocks) and β -D-mannuronic (M-blocks) acid residues as illustrated in Fig 2.8

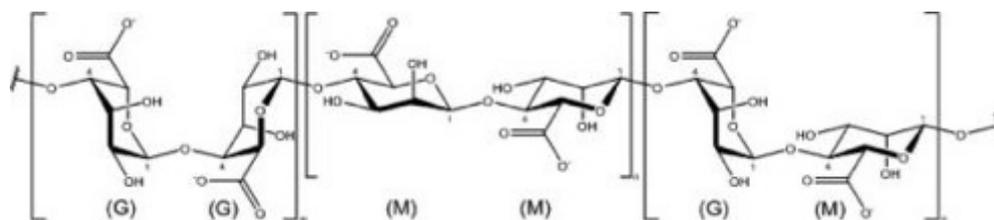


Fig. 2.8 Chemical structure of alginate

Both the arrangement of the monomers within the block structure and the size of the alginate affect the gel-forming ability, viscosity of the polymer and also three-dimensional macromolecular structures (Yian Wong et al., 2000). Since the gelation that permit the hardening of capsules made of alginate occurred by addition of divalent or monovalent ions (e.g. calcium ions) that link to the carboxylic residues of the G blocks, the higher the proportion of these, the greater the gel strength. The large use of alginate in microencapsulation is owing to the fact that it is an easy to prepare, cheap and safe material that easily forms gel matrices around the cells and mild process conditions (such as temperature) are needed for their performance; furthermore, especially when used in combination with chitosan it shows a gastric-resistant behaviour and it properly dissolves in the intestine and releases entrapped cells (Chávarri et al. 2012; Cook et al., 2013). Chelating compounds such as phosphate, citrate, EDTA and lactate, or other anti-gelling cations like Na^+ or Mg^{2+} which are naturally found in biological and bioprocessing environments can destabilize alginate gel, and in some cases trigger the total dissolution of gel network structure leading to the complete release of the encapsulated ingredients (Gombotz and Fong Wee, 1998; 2012). These chemical reactions are used in laboratory practice to obtain the artificial breakdown of alginates. However, alginate's major disadvantage is its inherent low mechanical stability especially in acidic environment. The mechanical properties of alginate microspheres can be enhanced by intensifying alginate concentration but this will lead to an exponential increase in its viscosity, which can limit extrusion of the alginate through the nozzle

(Whelehan and Marison 2011). For example, doubling the concentration of some alginate may result to a 10-fold increase in the viscosity of the alginate solution (Stark et al. 2002; Del Gaudio et al. 2005). For this reason, in order to increase the mechanical stability and protective ability of the capsules, alginate is usually used in combination with chitosan, mainly used as coating agent or in combination with other polymers used as filling agent (e.g. xanthan gum or Guar gum) added to alginate solution prior to the encapsulation process.

The second most abundant natural polymer after cellulose is the cationic polymer, chitosan. It is extracted as α -chitosan from chitin, the crab- and shrimp shells primary structural component (Shepherd et al. 1997). Chitosan is a linear co-polymer polysaccharide consisting of β (1–4)-linked 2-amino-2-deoxy-D-glucose (D-glucosamine) and 2-acetamido-2-deoxy-D-glucose (N-acetyl-D-glucosamine) units (Fig. 2.9). The proportion of N-acetyl-D-glucosamine and D-glucosamine residues usually determines its properties, biodegradability and biological role. This polymer can have diverse extent of diacetylation and it can be classified in terms of the percentage of primary amino groups in the polymer backbone, and molecular weight. Typical commercial chitosan usually has a DD of 70%-95%, and an Mw of 10-1000 kDa.

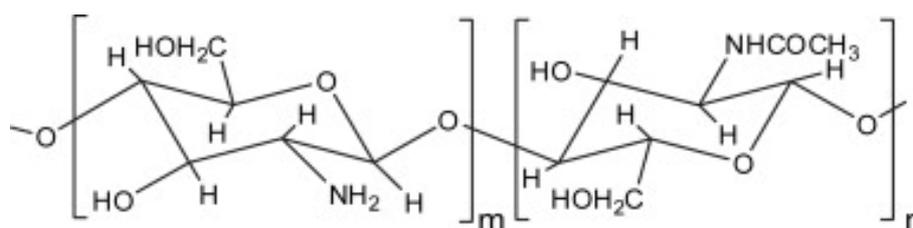


Fig. 2.9 Chemical structure of chitosan (George and Abraham, 2006).

Because of its unique polymeric cationic character of chitosan, electrostatic bindings between amino groups of chitosan and carboxylic groups of alginate can be formed obtaining strong gels with suitable physic-chemical characteristics. Furthermore for its good biocompatibility, non-toxicity and biodegradability, chitosan is gaining importance in the food and pharmaceutical field (Anal and Singh 2007). However, chitosan's antibacterial activity may pose a threat to the viability of probiotic bacteria. Due to this concern, and due that chitosan has a very good film-forming ability (Chávarri et al. 2012) but weak mechanical properties when used alone (Anal et al. 2003), chitosan is more used as an external shell in capsules made with anionic polymers like alginate (Anal et al. 2003) to form desired capsules (Riddle and Mooney 2004). This application of chitosan can improve the survival of the probiotic bacteria during storage and also in the gastrointestinal (GI) tract (Zhou et al. 1998; Capela 2006; De Prisco et al., 2015), and therefore, it is a good way of delivery of viable bacterial cells to the colon (Chávarri et al. 2010).

2.3 Microencapsulation of microbial cells

Thanks to the great versatility of microencapsulation due to the research and development of modern techniques and materials, this technology has been applied to many different microorganisms for as many purposes. As also showed in 2.1, even though the use of encapsulated cells have been explored also in fermentation and environmental

protection/decontamination, nowadays the sector of probiotic encapsulation seems to be the most attractive and of commercial success in food industry (Rathore et al., 2013).

2.3.1 Probiotics

According to the definition of the Expert Committee FAO/WHO (2001), probiotics are commonly defined as “live microorganisms which, when administered in adequate amounts, confer health benefits on the host”. Belonging mainly to Lactic Acid Bacteria (LAB), some probiotics have a long history of traditional use in the production of dairy fermented foods such as yoghurt, korut and kefir. To date, the latest evidences of their healthy effects on humans and animals are driving the modern forward looking research on probiotic functional properties in order to broader their application both in food and pharmaceutical field (De Prisco and Mauriello, 2016). That of probiotic health benefits is an area of intensive research in different domains that, according to the target sites and mechanism of action could be distinguished in intestinal and extra-intestinal (De Prisco and Mauriello, 2016). As a matter of fact, probiotics showed the best evidences of their efficacy in maintenance and restoration of the normal intestinal microflora and in the treatment and prevention of enteric infections and post-antibiotic syndromes in adults and children (Prantera et al., 2002; Sazawal et al., 2006). Additional evidences about the beneficial action of probiotic on the gastro-intestinal tract are related to the improvement of lactose metabolism (REF), prevention and treatment of inflammatory bowel disease and irritable bowel syndrome and to their efficacy as co-adjuvant for the current *Helicobacter pylori* eradication treatment (Camilleri, 2006; Hedin et al., 2007; Ruggiero, 2014). Among extra-intestinal effects, some promising outcomes in the use of probiotics have been individuated in the reduction of serum cholesterol level and blood pressure, reduction in the incidence of respiratory diseases, prevention of some cancers and treatment of urogenital diseases and bacterial vaginosis in women (Falagas et al., 2007; Khan et al., 2012). Although each probiotic effect is strictly related to a strain specific action and to a definite metabolic function, a general mechanism correlated to the beneficial action of probiotic has been delineated. This general mechanisms for probiotic *Lactobacillus* have been reviewed by Lebeer et al. (2008) and have been individuated in: a) adaptation mechanisms, related to probiotic cell ability of adaptation and colonizing the host; b) in probiotic mechanisms, mediated by an immunomodulatory effect (interaction and stimulation of host immune system), a microbe-microbe interaction at the bases of pathogen inhibition and in the epithelial barrier protection (Fig. 2.10).

2.3.1.1 Probiotic foods, health claims and labels

The reported evidences for the impact of probiotics on many end points of human health is driving the commercial development of products containing them (De Prisco and Mauriello, 2016). Indeed, probiotic food products comprise between 60 and 70% of the whole functional food market (Tripathi and Giri, 2014). The growing consumption of probiotic microorganisms includes foods (nutraceuticals or functional foods), food ingredients and supplements. It is common for probiotic products to be marketed on the premise that they have an important effect on the intestinal microbiota. Their most commonly reported claim “its consumption promotes the balance of the intestinal microflora” did not configure as a health claims rather than a structure/function claim because it is referred to a physiological

effect of the normal, not diseased, structure or function of the human body (Sanders, 2009). According to Rijkers et al. (2010) although a great number of health claims applications, often supported by high quality research on taxonomic, functional and clinical data, have been submitted at European Food Safety Authority (EFSA), no favourable opinion have been issued despite many biological evidences proved for many probiotic strains during *in vitro* and *in vivo* test. Also in the United States the Food and Drug Administration (FDA) has not yet approved any health claim for probiotic strains or products. Certainly, the scientific data to support health claims for probiotic products are often difficult to be collected and, consequently, to be provided (Farnworth, 2008).

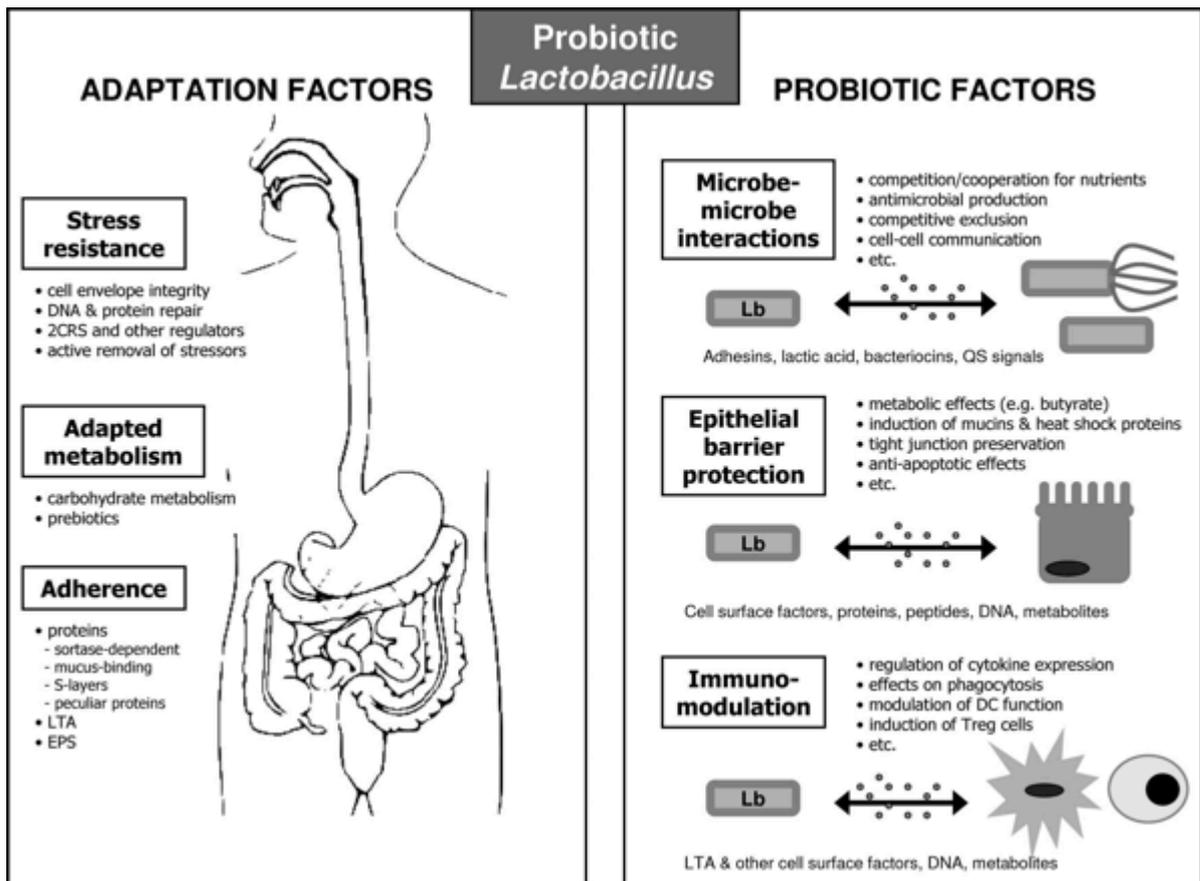


Fig. 2.10 General mechanisms of action of probiotic lactobacilli. Image by *Lebeer et al., 2008*.

It is also worth underline that the lack of proper evidence-based studies might be related to fact that clear guidelines to be followed by scientific research groups during the preparation of clinical trials are not still available. Instead, probiotic foods received FOSHU approval (Food for Specific Health Use) in Japan, thus they are regularly considered as products having beneficial effects on the physiological functions of the human body in maintaining and promoting health, and improving health-related conditions. In particular, foods carrying beneficial bifidobacteria and LAB have been included in the segment of foods helping the modification of gastrointestinal conditions. Health claims on FOSHU correspond to other function claims of the Codex Alimentarius or structure/function claim in the United States (Yamada et al., 2008) and, in alignment with foreign jurisdictions, claims on reduction of disease or disease risk are not allowed for probiotics. About the dose of assumption, no

specific cell load is recognized to guarantee a health effect even though a dose of 10^9 CFU/die is generally recommended. At this regard, it is paramount that cell probiotic load and composition comply with food labels. In fact, a serious weakness in the field of probiotic products is that the viability of the bacterial strains involved does not reflect what is reported on the label about the live microorganisms assumed with the food. From an exploration of the global market it raised that the currently employed probiotic strains exhibit little or no survival in final goods, showing cell loads lower than they are labelled, so the quality of the foods that carry them are still scarce (Corona-Hernandez et al., 2013).

2.3.1.2 Microencapsulation: a possible tool to overcome technological hurdles

A wide range of foods including fermented and non-fermented dairy products, ice creams and frozen desserts, fruit juices, peanut butter, cereal-based products, reduced fat (bio)spread have been enriched in probiotics to be evaluated as possible carriers of these beneficial microorganisms and to be placed on the market (Charteris et al. 2002, Klu et al., 2014; Molin, 2001; Sheu et al., 1993). To date, among the probiotic foods available on the market, fermented and non-fermented dairy products are still the most consumed by population. Most common probiotic strains added to foods belong to several species of *Lactobacillus* and *Bifidobacterium*, but also a yeast, *Saccharomyces cerevisiae* (*boulardi*), is used. Other genera (Enterococcus, Bacillus, and Escherichia) include strains recognized as probiotics but their main use is for food supplement production (Douglas et al., 2008; Santosa et al. 2006). Different factors that could affect microorganism behaviour and robustness into the different food environments have to be considered because they can interfere with the essential requirement of probiotics to achieve the intestine alive and metabolically active. Some are intrinsic, for example the type of culture selected, growth stage, subcellular injuries by heat or osmotic stress. The extrinsic factors are composition of food matrices, pH value, oxygen level, food manufacturing conditions and storage time (Reid et al., 2007; Soukoulis et al., 2014b). An example in Fig. 2.11 are reported the adverse factors affecting probiotic viability during cheese manufacture. Therefore, also the loss in probiotic viability during gastrointestinal transit, where the principal stressors are the shifting pH and bile, is to be considered as a hurdle that probiotics have to overcome to fulfil their biological role. At this regard, different strategies which improve probiotics technological and gastrointestinal robustness have been applied to get “super-fit” bacteria; careful selection of strains with highest native technological attitude, use of cell-protectants, strain adaption to a sub-lethal dose of a specific physical or chemical stress, genetic manipulation (Guidemond and Sánchez, 2012; Mills et al., 2011) as well as technological approach dealing with the inclusion of probiotics in edible films (Soukoulis et al., 2014a) or their microencapsulation (Anal and Singh, 2007; De Vos et al., 2010; Rokka and Rantamäki, 2010). Microencapsulation of bacterial cells has been described by some authors (O’Riordan et al., 2001; Anal and Singh, 2007; Sohail et al., 2012) and the main purpose, or at least, the most investigated aspect until now, is the protection of the cells under gastrointestinal conditions.

Microencapsulation of bacterial cells has been described by some authors (O’Riordan et al., 2001; Anal and Singh, 2007; Sohail et al., 2012) and the main purpose, or at least, the most investigated aspect until now, is the protection of the cells under gastrointestinal conditions. Yet despite, another challenge of microencapsulation is to protect probiotic cells in foods usually not considered as their vehicle such as bakery products (Malmo et al., 2013).

Microencapsulation, apart to the aforementioned effects, could offer many advantages in enhancing the handling of probiotic cultures as well as the masking of taste and aroma given by production of different metabolic compounds (e.g. acetic acid) produced during fermentation in foods where they are not required. Additional important aspect is the efficacy of encapsulation in improving probiotic survival after their inclusion in powdered formula where there is the detrimental effect of low water activity and oxygen exposure (Weinbreck et al., 2010).

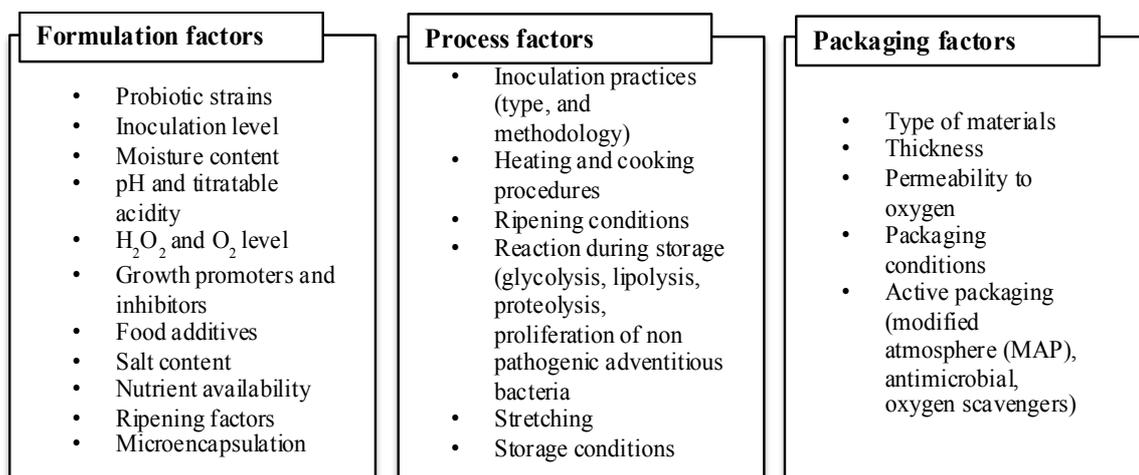


Fig. 2.11 Main factors affecting viability of probiotic bacteria in cheese. Image by *De Prisco and Mauriello, 2016*.

Selection of capsule materials as well as the technologies adopted in the fabrication of tailored probiotic microcapsules is of paramount importance because it strictly reflects the final morphological and functional properties of the capsules. Materials themselves play an important role in determining size, morphology, texture, porosity and other relevant properties of the capsules influencing the success in protecting or in targeting the delivery of probiotics. Certainly, food-grade biopolymers (i.e. alginate, chitosan, pectin, starch, carrageenan and milk proteins) are the most investigated and used matrices for cell encapsulation because of their effectiveness in protecting probiotics under several stress conditions (e.g. gastric pH, bile salts, enzymes) by working as real physical barriers or by exercising a buffered action, for example during the fermentation process. Their availability, low cost and biocompatibility are relevant requirements as well. Among the technologies applied for capsule production, emulsion, spray drying and extrusion are the most studied and applied on both laboratory and industrial scale. However, new technologies as complex coacervation (Bosnea et al., 2014) and vibrational extrusion technology (De Prisco et al., 2015) are emerging with promising results about cell entrapment efficiency, goodness of capsules in terms of morphological and functional properties as well as their scale up. Even though the microencapsulation is the main system to preserve probiotic viability against different stressors encountered during processing and storage of foods, few works investigate the functionality of microcapsules in protecting cells during their application in some foods (e.g. bakery and fermented meat products). May be as a consequence, non-dairy foods still represent only a niche in the market of probiotic foods. As recently reviewed by De Prisco and Mauriello (2016), the application of encapsulation to probiotic cells contributed to the research

development of conventional and non-conventional probiotic products. Some applications of encapsulated probiotics in bakery, meats, fruits and vegetables and dairy products have been reported in Tab. 2.2.

Table 2.2 Food products functionalized by addition of probiotic microcapsules within several food categories

Bakery Products
<ul style="list-style-type: none"> • <i>Bread</i> (Altamirano-Fortoul et al., 2012; Soukolis et al., 2014); • <i>Biscuits</i> (Reid et al., 2007); • <i>Chocolate soufflé</i> (Malmo et al., 2013); • <i>Stuff cakes</i> (Zanjani et al., 2012)
Meat derivatives
<ul style="list-style-type: none"> • <i>Fermented sausages</i> (Kearney et al., 1990; Muthukumarasamy and Holley, 2006; Sidira et al., 2014)
Fruit and vegetable juices
<ul style="list-style-type: none"> • <i>Raspberry</i> (Anekella and Orsat, 2013); • <i>Acerola nectar</i> (Antunes et al., 2013); • <i>Longan</i> (Chaikham et al., 2012 and 2013); • <i>Cranberry</i> (Doherty et al., 2012; Nualkaekul et al., 2013); • <i>Pomegranate</i> (Doherty et al., 2012; Nualkaekul et al., 2012); • <i>Berry</i> (Fратиanni et al., 2014); • <i>Carrot</i> (Ivanovska et al., 2014); • <i>Orange</i> (Krasaekoopt and Watcharapoka, 2014; Rodrigues et al., 2012); • <i>Peach</i> (Rodrigues et al., 2012); • <i>Apple</i> (Ying et al., 2013)
Dairy products
<p>Yoghurt</p> <ul style="list-style-type: none"> • <i>Plain yoghurt from bovine milk</i> (see De Prisco and Mauriello, 2016); • <i>Plain yoghurt from buffalo milk</i> (see De Prisco and Mauriello, 2016); <p>Cheese</p> <ul style="list-style-type: none"> • <i>Feta</i> (Kailasapathy and Masondole, 2005); • <i>Cheddar</i> (Amine et al., 2014; Fortin et al., 2011); • <i>White Iranian brined cheese</i> (Mirzaei et al., 2012; Zomorodi et al., 2011); • <i>White brined cheese</i> (Ozer et al., 2009); • <i>Kasar</i> (Ozer et al., 2008); • <i>Mozzarella</i> (Ortakci et al., 2012); • <i>Fiordilatte</i> (Minervini et al., 2012); • <i>Pecorino</i> (Santillo et al., 2014); • <i>Oxaca</i> (Rodríguez-Huezo et al., 2014).
Other food products
<ul style="list-style-type: none"> • <i>Ice cream</i> (Champagne et al., 2015; Homayouni et al., 2008); • <i>Mayonnaise</i> (Khalil and Mansour, 1998); • <i>Maize fermented beverage</i> (McMaster et al., 2005); • <i>Soured milk fermented beverage</i> (McMaster et al., 2005).

2.3.2 Starter Cultures

Despite the great interest reserved in the last years to the encapsulation of probiotics, microencapsulation of bacteria used to lead food fermentations or for the recovery of metabolites (e.g. ethanol, lactic acid) is another interesting subfield of cell encapsulation

(Westman et al., 2012). Both immobilization and microencapsulation of LAB have been suggested to improve fermentations in meat derivatives and dairy sector due to effect of preservation of cell viability, the reuse of starter cultures (Groboillot et al., 1994; McLoughlin and Champagne, 1994) and an easier recovery of cell-free metabolites. On the contrary, the use of microcapsules to improve the fitness of starter cultures and the comparison between fermentation performances of cells in free or microencapsulated form are still scarcely investigated. Similarly, also the study of the metabolism of microencapsulated bacteria is poorly explored. Encapsulation can influence cell growth and metabolism due both to the close contact among cells and to an increased diffusion resistance that may lead to nutrient-limited condition into the capsules (Westman et al., 2012;). Moreover, this condition could affect more strongly cells in the inner part of capsules rather than that located in the external part because of their increased contact with nutrients in the environment. In the same way, an increased contact with toxic substances, stressors and metabolites from fermentative process can involve cells on the outer layers of capsules, leading to their higher death rate. All this factors obviously can lead to variation in cell physiology. So, it is crucial to monitor the changes in the microenvironment as well as the external environment, and investigate their effects on the cells and on the biotechnological process of interest. Currently, few experiments have been carried out to explore physiology and metabolic behaviour of microencapsulated cells and most of them concern *Saccharomyces cerevisiae* and in general yeasts involved in ethanol production. To better understanding cell metabolism total carbohydrates, RNA, proteins, and two important carbohydrate reserves (glycogen and trehalose) of encapsulated *Saccharomyces cerevisiae* were analysed for the first time by Talebnia and Taharzadeh (2007). Also the fermentative capacity of encapsulated cells was monitored. They found that total cell proteins and RNA content decreased during 20 continuous fermentation cycles while ethanol production slightly increased. However, the most relevant finding is the discovering of higher accumulation in the cytosol of encapsulated cells of glycogen and trehalose, important reserved carbohydrates accumulated by cells when starvation and environmental stress occur. This corroborates the fact that encapsulated cells actuate some important metabolic changes. These modifications in metabolism surely are at the base of the enhanced cell viability that thus, is not only due to physical protection of capsules membrane that limits the diffusion of inhibitors. Two contributing reasons for increased tolerance were proposed by Westman et al., 2012a; i) the ability of cells in the cell pellet inside the capsule to convert inhibitors to less inhibitory compounds, leaving a less inhibitory medium to the interior cells, ii) the partially nutrient-limitation that interests encapsulated cell that triggers the environmental stress response which in this case is favourable since it likely increases the general tolerance toward further stress. These findings have also been supported by proteomic analysis revealing higher levels of several stress-related proteins as well as of proteins specifically important for the defence against inhibitors item of the study (Westman et al., 2012b). Food sectors that better exploited the advantages in the use of immobilized cultures are the ones of wine and brewing. In this context, the use of many yeast and lactobacilli strains in immobilized form permitted to production of alcoholic beverage with improved quality and sensorial properties where target metabolism such as the malolactic fermentation can be controlled (Nedovic et al., 2014). Finally, the current literature shows the potential of immobilization of cell cultures and of yeasts in particular as an important tool for the food sector, for carrying out optimized fermentations by high cell density, the improvement of metabolites production and reuse of biocatalysts as well as reduced risk of microbial contamination and physical and chemical

protection of the cells from fermentative harsh environment. Besides this premise, additional studies are needed to promote the industrial application of immobilized/encapsulated cells aimed at the developing of suitable and scalable encapsulation systems, the study of interaction between the precursors of the food matrices and cells and of related kinetics and finally the exploitation of encapsulated cell metabolism.

2.4 Market availability of microencapsulated cells for industrial application

According to a recent global industry analysis, the value of the global probiotic market, in strong growth from 2008, is predicted to get better until to exceed about 34 billions € (37.9 billions \$) in 2018 (Global Industrial Analysis, 2013). The most of foods (including beverages) available on the market are enriched with free probiotic bacteria while only a small part contains probiotics in microencapsulated form. Among the latter, especially supplements are formulated with microencapsulated cultures, although in the wake of the intense scientific research carried out in the last decades to promote the food industrial applications of microcapsules, it is likely to witness a remarkable growth of foods containing them. In a work of 2011, Champagne and Kailasapathy (2011) reviewed the situation of products available on the market delivering microencapsulated probiotics. Even though some notices about use of microcapsules from some food companies (Agropur, Kraft, Micropharma Inc. and Yoplait Inc.) for the functionalization of dairy product have been reported, to the extent of our knowledge, through an accurate exploitation on Internet, no products claiming to contain microencapsulated probiotics have been found. However, it should be highlighted that application of microcapsules in foods is not necessarily declared in label or advertised. Curiously, two products constituted by chocolate (ProBiotic bites) and cereals (YogActive plus) beads surrounding probiotic cells have been launched on the market. Obviously, because they appear like big size capsules they cannot be considered microcapsules. In this on-going scenario, the interest on the application of microencapsulation technology in food production from some enterprises is arising. Although many enterprises headquartered in Europe, especially Northern Europe, are mastering on probiotic microcapsules enhancement and selling, the application of microencapsulated probiotics in food is far from being a real industrial business. However, as above reported, it should be highlighted that functional food producers do not necessarily declare on labels the application of encapsulated probiotics. Vesale Pharma is one of the most known producers of probiotic food supplements, which on January 2011 filed the Intelicaps[®] patent, a technology based on gentle extrusion process for production of stable probiotic microcapsules having a diameter of 150-600 μm and promising to resist to many adverse conditions. Another one is the French Capsulae, a research and development enterprise that offers customized solutions in the field of microencapsulation. Even Lallemand, a well-known biomass producer enterprise, has a Health Solutions Division where a patented probiotic microencapsulation technology, the Probiocap[®], has been developed. It could be interesting to investigate the reasons why, although the extensive research and the presence of enterprises in the arena, the microencapsulation of probiotics is not usually practiced for developing of probiotic foods. Our opinion is that, like in other fields, more spreading of scientific results towards industry should be performed in order to offer for example new business chances in the production of desirable and currently unavailable probiotic foods like bakery products or instant hot beverages. Accordingly, the

European Patent n. 2451300 A1 describes compositions of heat resistant probiotic capsules. Furthermore, the invention relates to the health food products, particularly to bakery probiotic products. As already noted by Cheow and Hadinoto (2013), the method described in this patent seems to be too tricky and lengthy due to multiple coating procedures that can be costly upon scale-up. Proenol in Scott Laboratories developed ProElif[®], an encapsulated yeast product for sparkling wine production. Yeasts are encapsulated in double alginate layer and are sold on the premise that they can be directly inoculated into the bottle, eliminating the need to prepare a starter culture, ensuring the control of the number of cells per bottle and facilitating cells removing from wine. ProRestart and ProDessert are other commercialized encapsulated yeasts for winery industry. These products contain yeast cells encapsulated in alginate that are also acclimatized to alcohol and other harsh conditions. Unfortunately, no data about commercial availability of more encapsulated starter culture seem to be available.

CHAPTER 3

Validation of microencapsulation of living cells by vibrating technology

3.1 Introduction

Microencapsulation of living cells is becoming a very diffuse technique in several fields of biosciences including medicine (Blasi et al., 2013), nutraceutical sciences (Burgain et al., 2011) and food technology (Rodrigues et al., 2012; Malmo et al., 2013). Particularly, in the field of probiotic cells, microencapsulation serves as a strategy to i) improve their viability during their processing and storage, ii) to protect their viability and metabolic activity during GI transit and iii) to contribute to the development of new functional food products. Despite of a wide choice of encapsulation technologies and materials, living cells require gentle techniques and one of this is the extrusion (De Vos et al., 2010). This last is evolved in the vibrating technology, as recently described by Whelehan and Marison (2011). This is an almost new technology, developed before in the Inotech laboratories and then in the Buchi ones. Even though this technique was used for the first time in 1985 by Hulst et al. (1985) and some other authors used it in the last three decades (Brandenberger and Widmer, 1998; Serp et al., 2000; Mazzitelli et al., 2008; Dorati et al., 2013), only few works (Martoni et al., 2008; Shi et al., 2013) described the microencapsulation of bacterial cells by using the vibrating technology. The aims of this work were to assess the effectiveness of bacterial cell microencapsulation using vibrating technology, to evaluate the cell viability during the storage of fresh and freeze-dried microcapsules and to investigate some of the probiotic and technological properties of probiotic strains after microencapsulation by vibrating technology. At this purpose *Lactobacillus reuteri* DSM 17938 was selected as probiotic strain model.

Lactobacillus reuteri DSM 17938 is a generally recognized as safe (GRAS) probiotic strain deposited by BioGaia in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and referenced in the scientific literature also as SD 2112, ING 1 and MM53. *Lb. reuteri* DSM 17938 derives by the depletion of two plasmids encoding for antibiotic (lincomycin and tetracycline) resistance (Roos and Rosander, 2005; Rosander et al., 2008) from the strain ATCC 55730, isolated in Perù from human breast milk (Johnson et al., 2006). This probiotic strain is characterized as gastro-intestinal resistant (Wall et al., 2007) and it was found to encode for protein MapA, a mucus adhesion promoting protein, and able to bind mucus and intestinal epithelial cells Caco-2 (Ouwehand et al., 2001; Miyoshi et al., 2006). Later, in a study of 2007, it was demonstrated that calcium ions significantly increased the adherence of *Lb. reuteri* to intestinal mucosa, suggesting the strategic inclusion of this strain in milk-based formula (Larsen et al., 2007). Furthermore, its selective ability in binding intestinal cells of IBD affected patients, more than that affected from diverticulitis or rectal carcinoma (Ouwehand et al., 2003) encouraged the selective use of probiotic strains in the treatment of specific syndromes. The probiotic effect of *Lactobacillus reuteri* DSM 17938 has been widely

investigated both *in vitro* and *in vivo* on animals and humans (adults and childrens). Similarly to other *strains* of this species, *Lb. reuteri* DSM 17938 is a producer of antimicrobial substances (reuterin) and its consumption has been correlated to a stimulation of immune system ad exemplum through i) increasing of anti-inflammatory interleukin 10 in pregnant women (Jacobson et al., 2005); increasing of B and T-lymphocytes and decreasing of gastric mucosal histiocytes (Valeur at al., 2004); iii) reducing fecal calprotectin (Mangalat et al., 2012). Besides the efficacy on adults in the suppression of *H. pylori*, reduction of gingival inflammation and plaques and IBS symptoms (Niv et al., 2005; Krasse et al., 2005; Caglar et al., 2006), *Lb. reuteri* DSM 17938 is highly recommended for paediatric use beyond its proved efficacy in limiting the relapse to eczema in infants related to cow's milk consumption, reducing acute diarrhoea and diarrhoea from rotavirus in children and in other conditions (Szajewska et al., 2014; Urbanska and Szajewska, 2014). Since also it's safety and tolerability has been widely confirmed *in vivo* (Wolf et al., 1995, 1998; Mangalat et al., 2012), this probiotic strain is included in many pharmaceutical formulations (BioGaia ProTectics drops, BioGaia ProTectics chewing gum, BioGaia ProTectics straws) while its spreading in foods is still only confined to research level.

3.2 Methodologies and materials

3.2.1 Microorganisms and culture conditions

Lactobacillus reuteri DSM 17938 was isolated from Reuterin[®] (Noos S.r.l.; BioGaia AB, Stockholm, Sweden) and cultured in MRS Broth (OXOID Ltd., Basingstoke, Hampshire, England) at 37°C, checked for purity and maintained on MRS Agar (Oxoid). Free and microencapsulated cells of *Lactobacillus reuteri* DSM 17938 were routinely cultured and counted on MRS Agar at 37°C for 48 h in aerobic conditions. *Pseudomonas fragi* 25P used in reuterin production test, belonging to microorganisms collection of Department of Agriculture, University of Naples Federico II, was previously isolated from fresh meat sample (Ercolini et al., 2009). It was cultured in Tryptone Soya Broth (TSB, Oxoid) supplemented with 5 g/L Yeast Extract Powder (Oxoid) at 20°C.

3.2.2 Encapsulation of *Lactobacillus reuteri* DSM 17938

Microencapsulation of bacterial cells was carried out by using the Encapsulator B-395 Pro equipped with an 80 µm nozzle and a syringe pump (BÜCHI Labortechnik, Flawil, Switzerland). In detail, cells from a defined volume of *Lactobacillus reuteri* DSM 17938 culture in the early stationary phase were harvested by centrifugation at 5200 g for 15 min. Cell pellet was washed once in an equal volume of a sterile quarter-strength Ringer solution (Ringer), harvested by centrifugation and finally suspended in an equal volume of a 20 g/L alginate (Sigma, Milan, Italy, product n. A2033) solution, previously degassed and sterilized, to reach a concentration of about 9.40 ± 0.10 Log. The syringe, used in the feeding system, was loaded with 50 ml of the alginate cell suspension and placed on the Encapsulator according to the instruction of supplier. The microencapsulation conditions used were: flow rate 2.91 ml/min, vibration frequency 1740 Hz, electrode voltage 950 mV. Alginate droplets containing bacterial cells were hardened in 200 ml of a 0.5 mol/L CaCl₂ solution (in ratio 4:1 with alginate cell suspension) for about 20 min in stirring to obtain monodisperse cross-linked microcapsules.

Suspension was left 30 min at room temperature for the sedimentation of microcapsules and then a volume of 150 ml of the upper phase was gently sucked and discarded to restore the initial cell concentration. Alginate microcapsules (AA) were routinely stored at 4°C for further experiments.

3.2.3 *Coating of alginate microcapsules with chitosan*

A 7 g/L chitosan solution was prepared dissolving Chitosan (Sigma, product no. 448869) in a 0.14 mol/L acetic acid solution (pH 3.2) under stirring at 8000 rpm for 20 min at 50°C. The solution was sterilized at 121°C for 15 min. A were added 1:10 (w/v) and stirred at 4000 rpm for 15 min to obtain, after sedimentation and gentle draining of supernatant, chitosan-alginate microcapsules (AM). They were suspended in Ringer to rich the same volume of A sample and routinely stored at 4°C.

3.2.4 *Freeze drying of alginate microcapsules*

Pellets from A and M after discarding excess of CaCl₂ solution were diluted in a volume of sterile skimmed milk (Oxoid) to restore the cell concentration of alginate-cell suspension. It was frozen at -18°C overnight and then subjected to lyophilization process (Thermo Scientific Heto PowerDry PL6000 Freeze Dryer). For scanning electron microscopy (SEM) analysis skimmed milk was replaced with 50 g/L trehalose and 50 g/L sucrose solution (Chen and Mustapha, 2012). Freeze-dried AA and MM are below referred as FAA and FMM, respectively. For cell counting of freeze-dried microcapsules they were before resuspended in Ringer in order to make the initial solid content.

3.2.5 *Entrapment efficiency (EE) of microencapsulation process*

Cell loading was determined on integer and disrupted fresh and lyophilized AA and MM. Microcapsules were serially diluted in Ringer or 0.5 mol/L phosphate buffer solution pH 7.0 (Gombotz and Wee, 1998) and plate-counted. Entrapment efficiency (EE) was calculated by dividing the viable count of disrupted microcapsules by the cell load before the microencapsulation (Graff et al., 2008). Furthermore, we calculated for all samples the average number of CFU for each microcapsule with the formula: $N = N_d / N_i$, where **N** is the average number of CFU for each microcapsule; **N_d** is cell count (CFU/ml) of disrupted microcapsules; **N_i** is cell count of integer microcapsules. We supposed that each colony is derived from a single integer microcapsule and therefore the measure unit of N_i (CFU/ml) may be replaced from microcapsules/ml.

3.2.6 *Sizing of microcapsules by light microscopy*

Samples were analysed by using a Zeiss Axiovert 135 light microscope (Carl Zeiss S.p.A., Arese, MI, Italy) at x 320 and x 200 magnification and a calibrated micrometer. About 20 microcapsules from each encapsulation trial were analysed immediately after the process, during the storage at different conditions and after each treatment described below.

3.2.7 *Scanning electron microscopy (SEM) analysis of microcapsules*

Samples were analysed with a scanning electron microscopy (SEM-Evo 40, Carl Zeiss, Oberkochen, Germany) to examine the surface morphology of microcapsules. AA and AM were rinsed three times with MilliQ water (Lichrosolv Water for Chromatography) and then 10 µl of the sample was placed on a pin type SEM specimen mount and maintained at 42°C for 1 h in order to reach a gentle dehydration of the microcapsules and their fixing. FAA and FAM were directly placed on the SEM specimen mount previously coated with a double sided tape to fix the microcapsules. All samples were sputter-treated in a metallizer (Agar Sputter Coater) with gold-palladium to reach a thickness of coating of 100 Å and then observed in high vacuum mode.

3.2.8 Evaluation of cell viability during storage of alginate matrix microcapsules

Pellet from AA after discarding excess of CaCl₂ solution was diluted in a volume of different sterile solutions to restore the cell concentration of alginate-cell suspension. Solutions were: i) Ringer, ii) 9 g/L sodium chloride (NaCl) and iii) 0.5 mol/L calcium chloride (CaCl₂). Samples were stored for 28 days at 4 and 20°C and viable counting was performed on MRS agar at 0, 7, 14, 21 and 28 days. Cell load was determined after disruption of microcapsules. Free cells were managed in the same way and used as control sample.

3.2.9 Simulated gastrointestinal (GI) conditions

A 5 g/L sodium chloride saline solution added with 3 g/L pepsine (Sigma, product no. P7000) at pH 2.5 was prepared (gastric simulating solution, GSS). Intestinal condition was simulated with a 5 g/L bile (Sigma, product no. B8381) solution (ISS, intestinal simulating solution). Both solutions were sterilized by filtration (0.22 µm). One ml of AA and AM was suspended in 9 ml of GSS and ISS and incubated at 37°C with shaking by hand each 30 min for 3 and for 4 h, respectively. After treatment samples were plate counted after their disruption. Free cells were used as control.

3.2.10 Osmotic stress conditions

The resistance of free and microencapsulated *Lactobacillus reuteri* DSM 17938 cells in osmotic stress condition was evaluated by using a medium with a high sugar concentration. In particular, 100 g of apricot jam were inoculated with 1 g of AA or AM. Samples were stored at 4°C and analysed for viable count immediately after the adding and after 3 h. Free cells, used as control, were decimally serially diluted in Ringer, while AA and AM in 0.5 mol/L phosphate buffer pH 7.0, both supplemented with 100 g/L sucrose (Sigma) and plate counted.

3.2.11 Reuterin production assay

An antagonistic deferred agar spot test was carried out to detect reuterin production against *Pseudomonas fragi* 25P. Briefly, 10 µl of free cells and microencapsulated cells were spotted on MRS Agar supplemented with glycerol (250 mmol/L) and incubated at 37°C for 24 h. After cell growth, the plates were covered with 10 ml of TSB supplemented with 7,5 g/L agar and 5 g/L yeast extract seeded with 2% of an overnight culture of the *Pseudomonas fragi* 25P. After the incubation at 20°C for 24 h, the antimicrobial activity was observed as a growth inhibition

zone of the indicator strain.

3.2.12 *Monitoring of cell damage by a fluorescence microscopy test*

Free and microencapsulated *Lactobacillus reuteri* DSM 17938 cells were dyed by using a LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes, Eugene, Oregon, USA) to investigate cell membrane damage at different steps of the microencapsulation process and after the exposure to previously described stress conditions. Samples were stained and visualized by epifluorescence microscope according to the procedure followed by Malmo et al. (2013). It was possible to distinguish between damaged and non damaged cells, stained in red and green, respectively. Images were captured by a Nikon Coolpix 4500 Digital Camera equipped with a microscope adapter.

3.2.13 *Data analysis*

Analysis was carried out in triplicate and the cells viability after microencapsulation was calculated as average of five independent experiments. A Two-way Anova test and a t-test analysis (Microsoft Excel for Mac version 11.5) were performed to ascertain significant differences between averages; significance was declared at $P < 0.05$.

3.3 Results and discussion

3.3.1 *Entrapment efficiency (EE) and bacterial survival*

The bacterial load before and after microencapsulation was evaluated to assess the efficiency of microencapsulation experiments (Table 3.1). Results of viable counts of disrupted AA revealed a reduction in the cell load with respect to the alginate-cell suspension of only 0.35 Log cycles suggesting a high EE value (Table 1). This is corroborated by a cell count of about 10^3 CFU/ml which was recovered in the CaCl_2 supernatant of AA (data not shown). Viable cell counts from integer and disrupted AA were significantly higher ($P < 0.05$) compared to counts from AM samples (Table 1). CFU for each microcapsule was estimated to be 99 for AA and 92 for AM (Table 1). In addition, the bacterial load of lyophilized microcapsules showed 100% survival immediately after the freeze drying process (Table 3.1). Verification of the integrity of cell membrane of the microencapsulated cells following the staining reaction by fluorescence microscope (Fig. 3.1) show that all the cells in both AA and AM were green thus indicating lack of damage.

3.3.2 *Morphology of microcapsules*

Size of AA and AM was evaluated immediately after their production and during the storage in different conditions (only for AA). Optical microscopy images are reported in Fig. 3.2. They show microcapsules with an average diameter (\pm standard deviation) of $110 \pm 5 \mu\text{m}$ in both samples, suggesting that chitosan coating did not lead to a significant variation in microcapsule bead diameter. Moreover, size of AA remained unchanged during their storage in different conditions (data not shown). Images show microcapsules regular in shape and without agglomeration among them and the border-line of AM less regular compared to that of AA.

Tab. 3.1 Bacterial load (Log CFU/ml) of microcapsules before (AA and AM) and after (FAA and FAM) freeze-drying obtained by using a cell/alginate suspension of 9.40 ± 0.10 Log CFU/ml and parameters of microencapsulation yield.

Microcapsule samples	Intact	Disrupted	Parameters		
			EE (%)	N (CFU/microcapsule)	Survival after drying (%)
AA	7.14 ± 0.20^A	9.15 ± 0.14^A	97	99	-
AM	6.89 ± 0.15^B	8.82 ± 0.22^B	-	92	-
FAA	7.27 ± 0.23^A	9.19 ± 0.13^A	-	99	100
FAM	6.81 ± 0.18^B	8.92 ± 0.20^B	-	92	100

* Mean results of five independent trials \pm standard deviation. Different indices within the same column indicate a significant difference ($P < 0.05$).

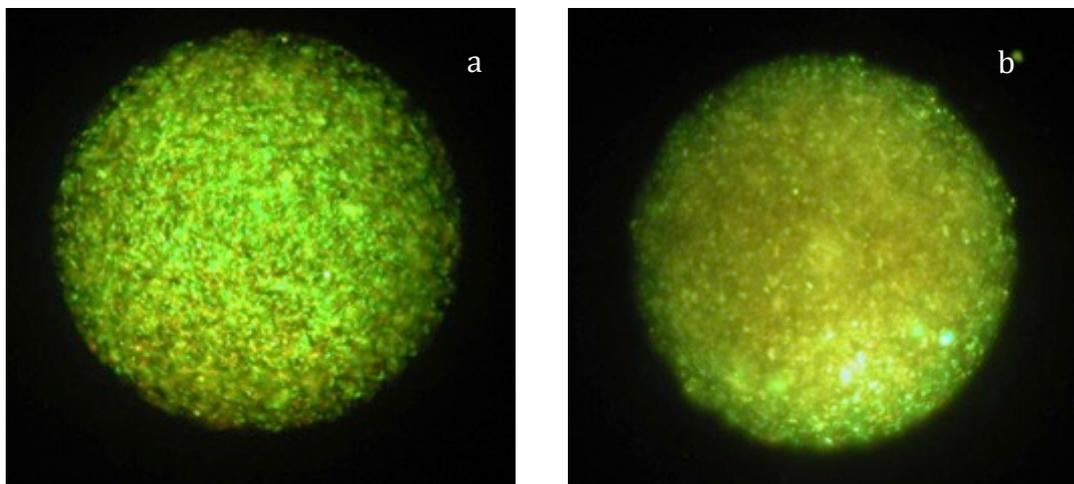


Fig. 3.1 Fluorescence microscopy images at x 400 magnification of stained *Lactobacillus reuteri* DSM 17938 in alginate, AA (a) and chitosan-alginate, AM microcapsules (b).

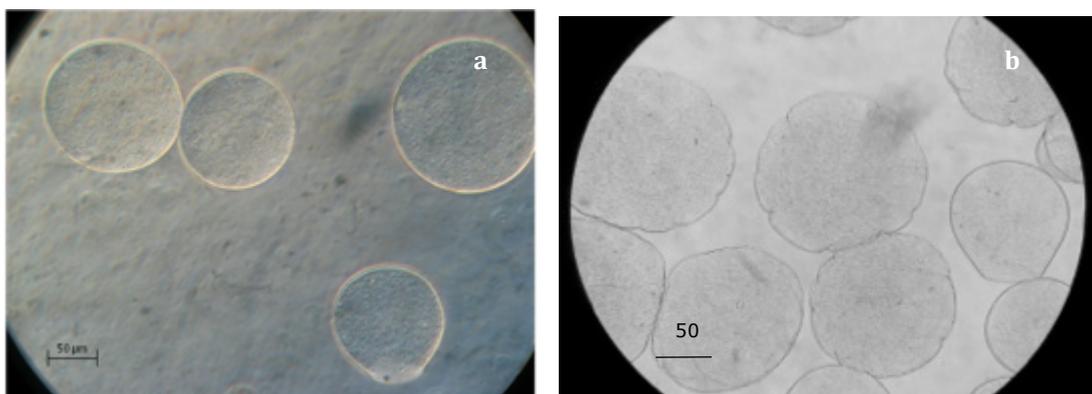
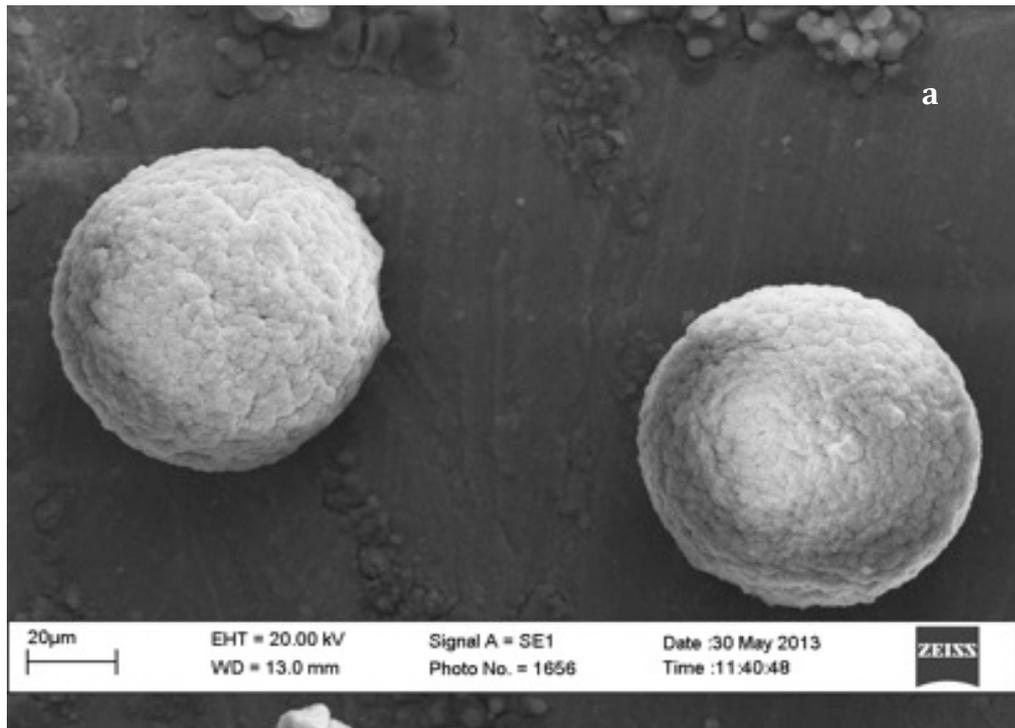


Fig. 3.2 Optical microscopy images of alginate microcapsules, AA at x 200 magnification (a) and of chitosan coated alginate microcapsules, AM at x 320 magnification (b).

SEM images show that AA were perfectly spherical in shape, rough and continuous in surface without hollow zones or cracks (Fig. 3.3a). Occasionally, collapsed microcapsules were

visualized (Fig. 3.3b). As shown in Fig. 3.4a, chitosan coating led to a little modification of microcapsules shape that appeared wrinkled and rougher compared to AA. The surfaces of AM samples were also found to be continuous without any observable hollow or cracks. On the other hand, increased roughness and more marked spongy-like features were observed on the surface of AM (Fig. 3.4b) compared to that of AA (Fig. 3.3c).

Both microcapsules were found to retain their shape after freeze-drying (Fig. 3.5a and 3.6a). However, the surface of FAA (Fig. 3.5b) appeared more plain and skinny than the case before freeze-drying (Fig. 3.3c) while FAM surface roughness remained fairly the same after freeze drying (Fig. 3.6b).



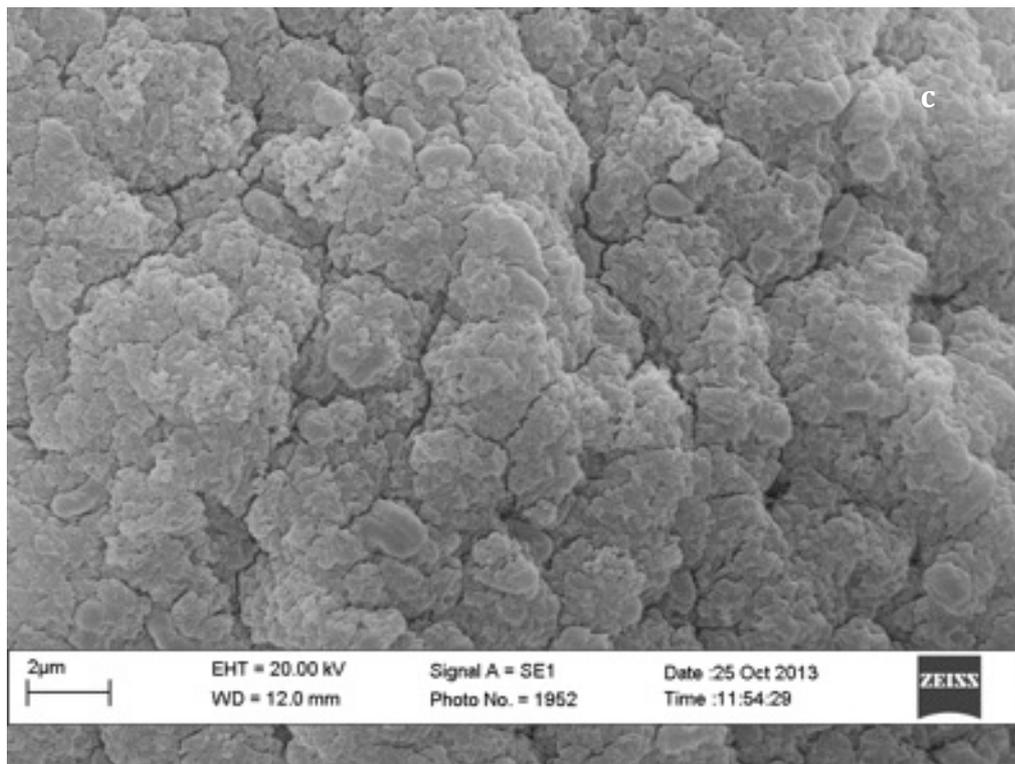
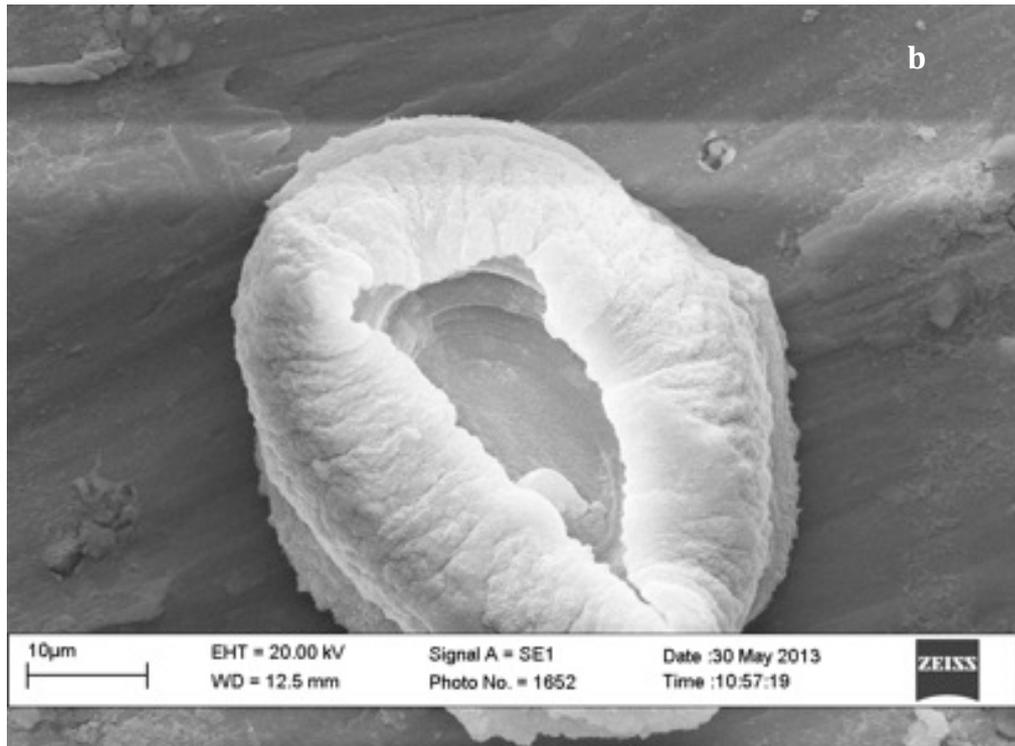


Fig. 3.3 SEM images of alginate microcapsules (AA). a) AA with a characteristic morphology at x 1600 magnification; b) collapsed AA at x 4300 magnification; c) particular of AA surface at x 15000 magnification.

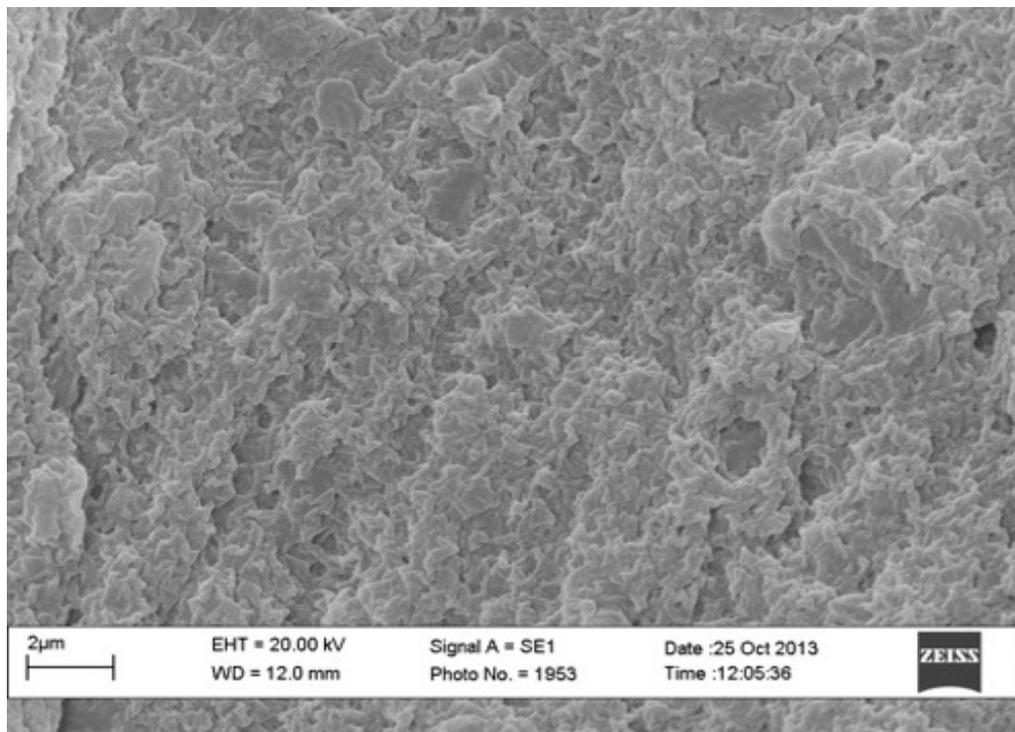
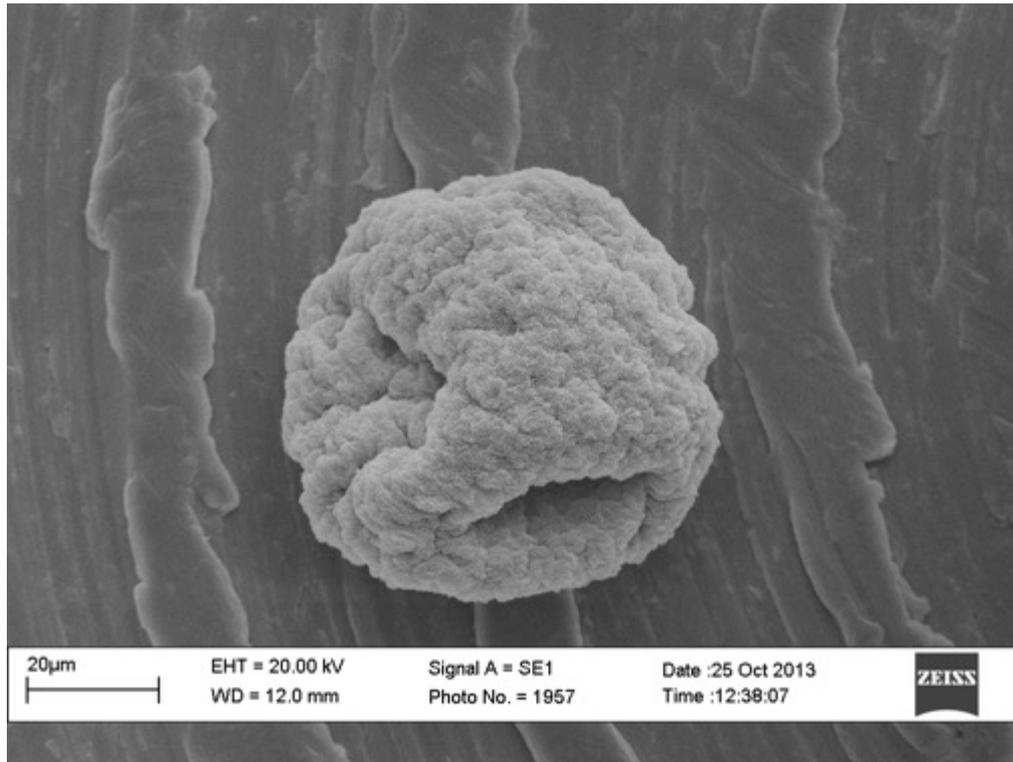


Fig. 3.4 SEM images of chitosan-alginate microcapsules (AM). a) AM with a characteristic morphology at x 240 magnification; b) particular of AM surface at x 15000 magnification.

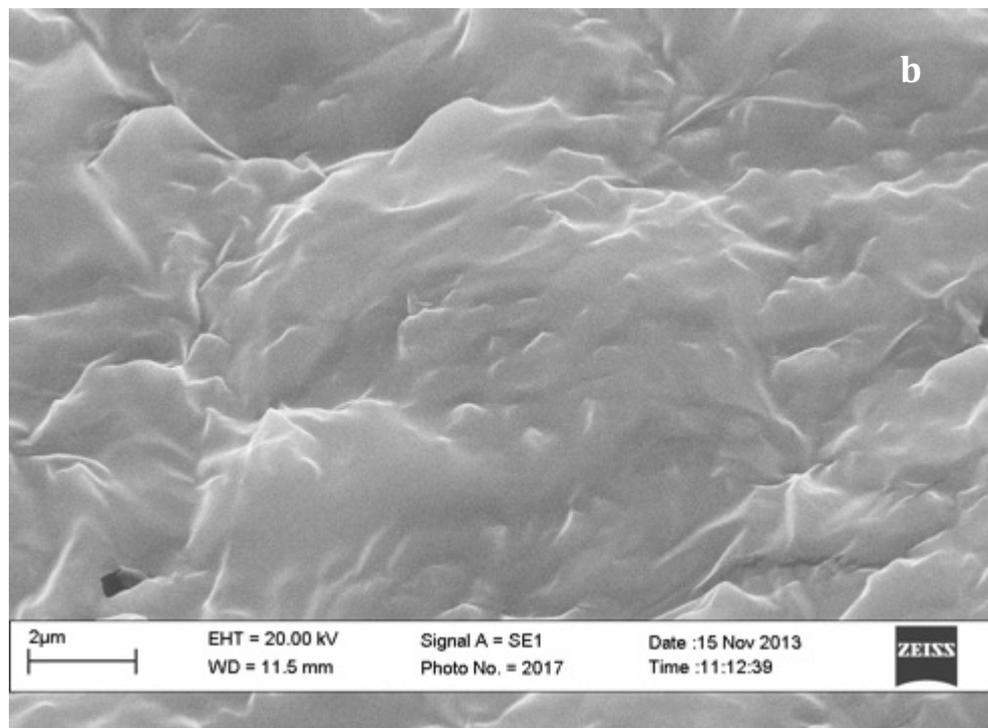
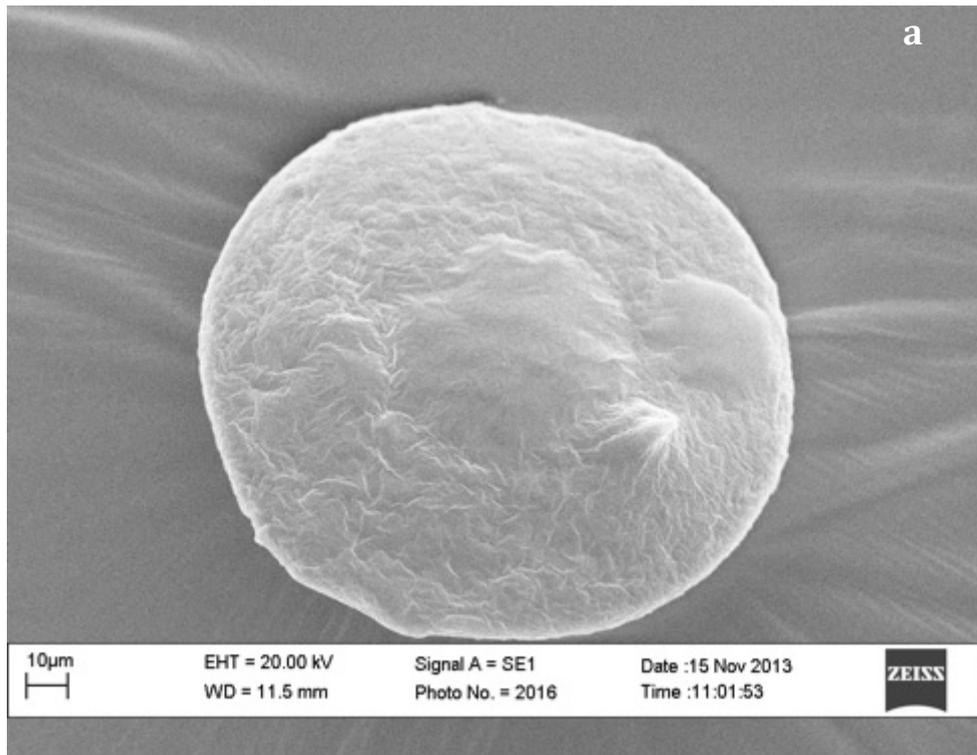


Fig 3.5 SEM images of freeze dried alginate microcapsules (FAA). a) FAA with a characteristic morphology at x 2400 magnification; b) particular of FAA surface at x 15000 magnification

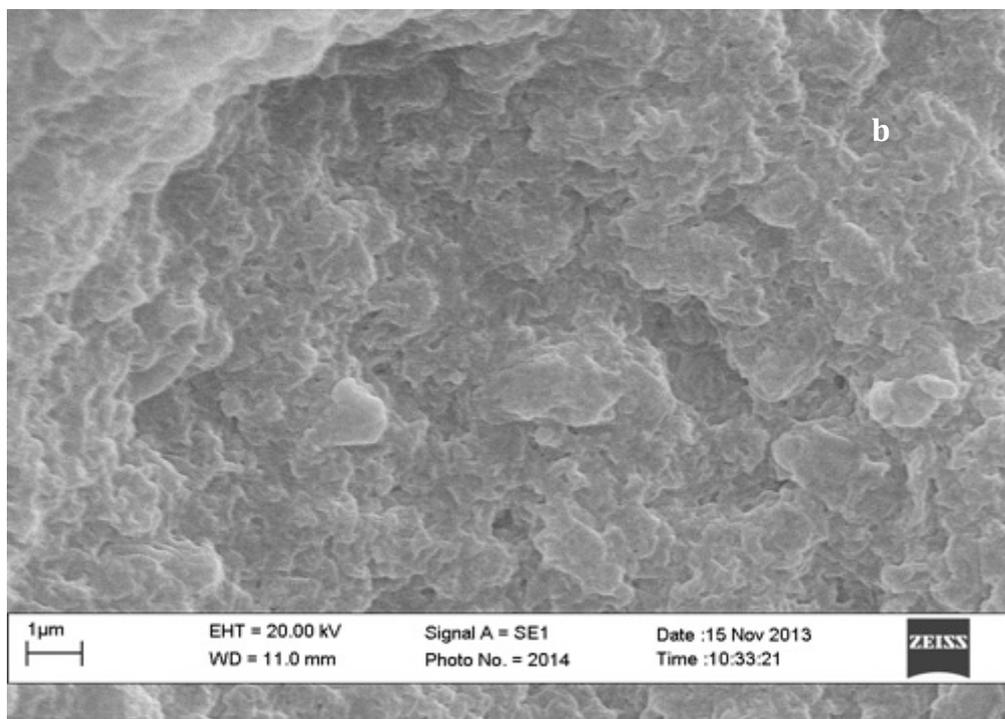
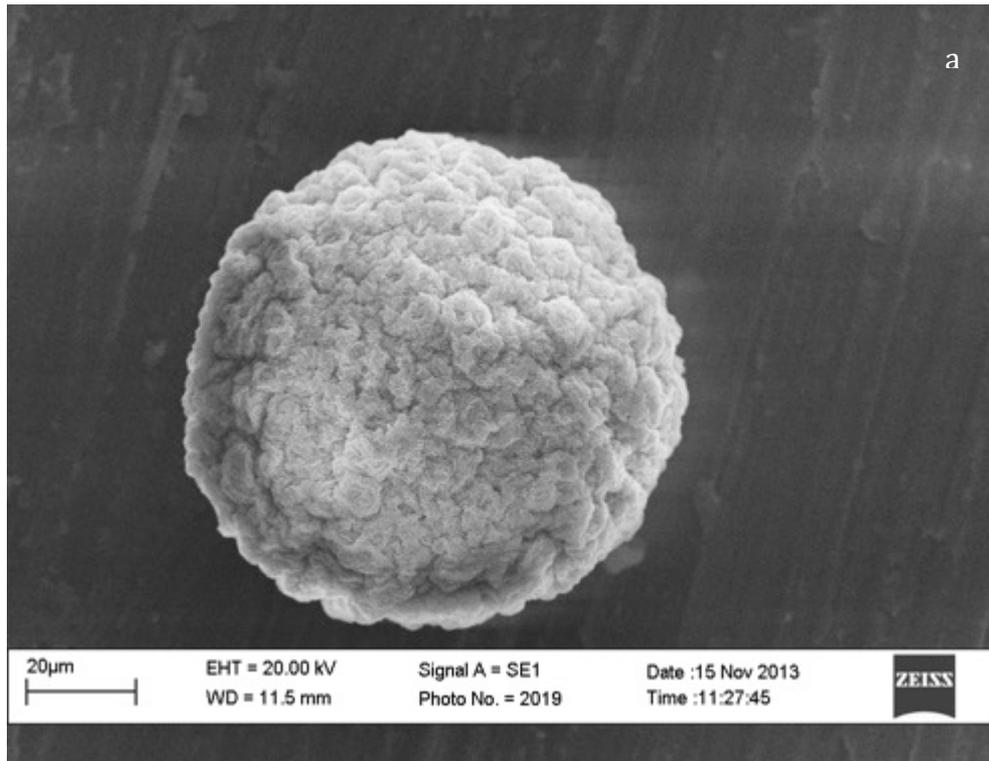


Fig. 3.6 SEM images of freeze dried chitosan-alginate microcapsules (FAM). a) FAM with a characteristic morphology at x 2400 magnification; b) particular of FAM surface at x 15000 magnifications.

3.3.3 Survival of probiotic cells during storage of A

Survival kinetics of free and microencapsulated *Lactobacillus reuteri* in alginate matrix stored in

different conditions are reported in Fig. 3.7. The storage at 20°C led to a dramatic decrease in viability for both free and microencapsulated probiotic cells. Total reduction in the cell load was observed on the 14th day of storage for free cells in CaCl₂ and on the 21st day of storage for all other samples except for A in NaCl, where total cell reduction was observed at the end-point of the storage period (Day 28: Fig. 3.7a). Different results were obtained for samples stored at 4°C (Fig. 3.6b). Best microcapsule preservation was achieved for A stored in Ringer and in NaCl for which only 1 Log reduction in viable cell counts ($P < 0.05$) was observed after 28 days in both cases. On the contrary, viable count of free cells in the same solutions decreased by about 3 Log cycles at the end of storage. Furthermore, a reduction of 3 Log cycles in viable cell count was registered for A in CaCl₂ while about 4.5 Log reduction was observed in the case of free cells instead (Fig. 3.7b).

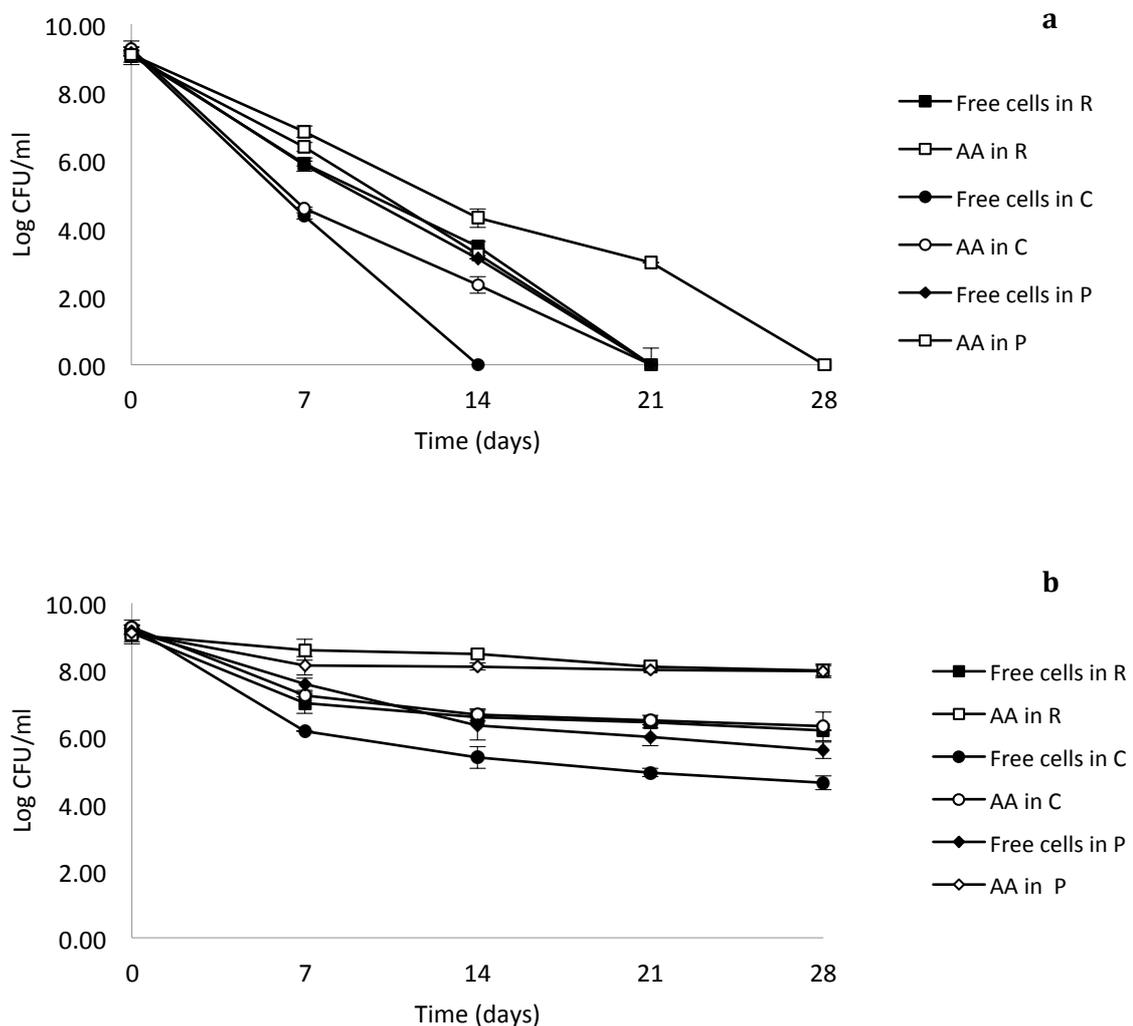


Fig. 3.7 Survival kinetics of free and microencapsulated *Lactobacillus reuteri* DSM 17938 in alginate capsules (AA) stored in Ringer (R), NaCl (P) and CaCl₂ (C) at 20°C (a) and 4°C (b) along 28 days. Differences between mean values were analysed by two-way ANOVA. Significance was declared at $p \leq 0.05$. The error bars represent standard deviations.

3.3.4 Gastrointestinal (GI) stress conditions

The results of viable counts and survival percentage of free and microencapsulated *Lactobacillus reuteri* cells after exposure to simulated GI conditions are shown in Fig. 3.8.

Results show that free cells were very sensitive to GSS. AA significant ($P<0.05$) reduction of 2.09 Log cycles was observed after 3 hours, resulting in a percentage survival of less than 1%. On the contrary, treatment of free cells for 4 hours in ISS led to a reduction of only 0.82 Log cycles which translates to about 16% of cell survival. Both AA and AM conferred resistance to cells in GI conditions ($P<0.05$) with exception of A in GSS where a significant ($P<0.05$) but moderate cell count reduction of about 0.35 Log cycles was observed.

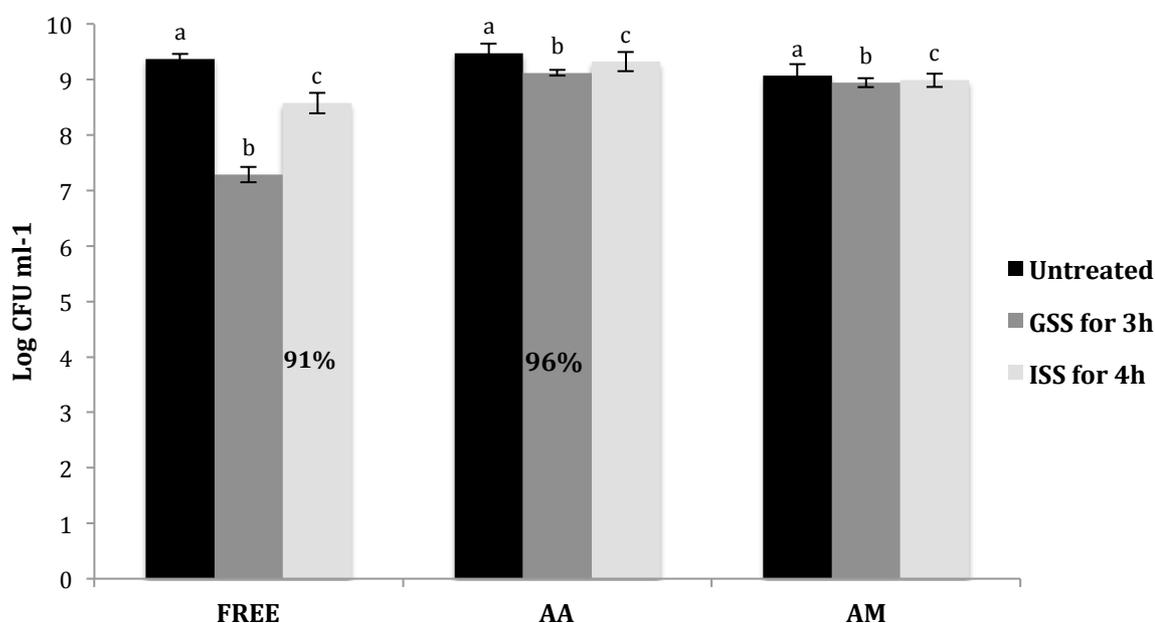


Fig. 3.8 Viable counts and survival percentage of free and microencapsulated *Lactobacillus reuteri* DSM 17938 before and after incubation in GSS for 3h and in ISS for 4h. The error bars represent standard deviations. Different letters labeling bar graphs of the the sample categories indicate that mean values are significantly different ($p\leq 0.05$) as determined by t-test.

Results of viable staining of both microcapsule types after GSS (Fig. 3.9a and 3.9b) and ISS treatments (Fig. 3.9c and 3.9d) are depicted in Fig. 3.9. Generally, microcapsules maintained their shape and size features. However, after GSS treatment a modest but clear reduction in size of AM, as well as their aggregation was observed (Fig. 3.9b). Epifluorescence microscopic images showed that most of the cells were green-stained for all samples although in a few cases, zones of some capsules appeared light red. However, this red staining was much less marked than observed in the case of the free damaged cells (data not shown). The presence of free cells indicates a slight release of cells from both microcapsules when they were exposed to the GSS (Fig. 3.9a and 3.9b). Finally, green stained debris were clearly visible after 4 hours of exposure of AM to ISS (Fig. 3.9d).

3.3.5 Osmotic stress condition

Results of this experiment are reported in Fig. 3.10. It was observed that both microcapsules were able to protect probiotic cells from osmotic stress. In fact, at time zero (T_0), a reduction of 1.2 Log cycles was observed for free cells while only 0.6 Log reduction was registered in the case of microcapsules. A further significant ($P<0.05$)

reduction in cell viability was registered at T3 for free cells (0.7 Log cycles) and AM (0.4 Log cycles) while no variation was found in the case of the AA. Hereafter, subjecting the microcapsules and free cells to osmotic stress beyond 3 h led to a significant ($P<0.05$) over reduction of viable cell count by about 2 Log cycles for free cells and by about 0.67 and 1 Log cycle in the case of AA and AM, respectively.

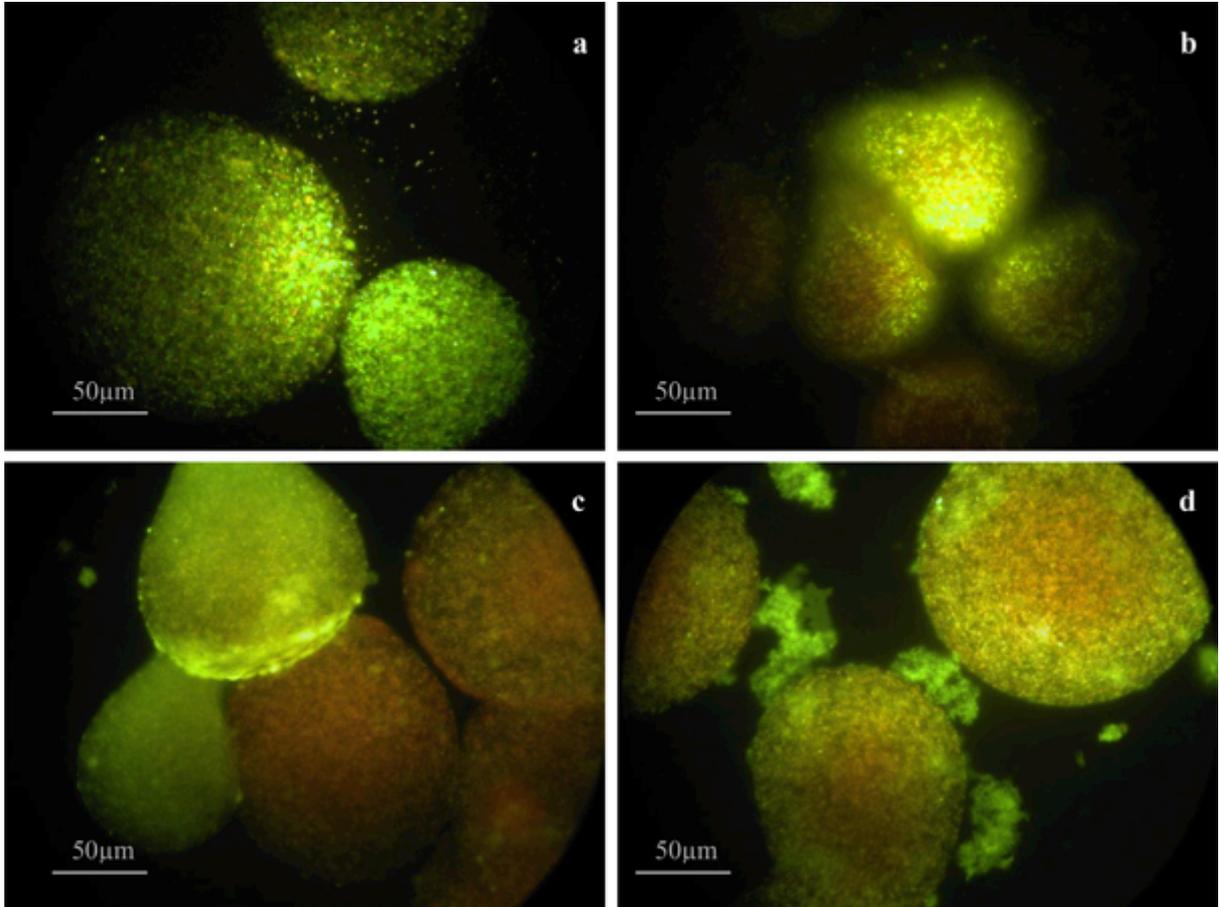


Fig. 3.9 Fluorescence microscopy images at x 400 magnification of viable staining of microencapsulated *Lactobacillus reuteri* DSM 17938 after treatment in gastrointestinal conditions. Alginate microcapsules, AA (a) and chitosan coated alginate microcapsules, AM (b) after 3 h in GSS, AA (c) and AM (d) after 4 h in ISS.

3.3.6 Reuterin production

Results of reuterin test showed that neither alginate microencapsulation nor further chitosan-coating of AA affected the antimicrobial substance production by *Lactobacillus reuteri*. The AA and AM showed an inhibition halo of the indicator strain by 38 and 31 mm, respectively, compared to 42 mm produced by free cells.

3.4 Discussion

Extrusion, emulsion and spray drying are the main techniques reported in literature for the microencapsulation of probiotic bacteria. Emulsion technique is easy to scale up but the main disadvantages are the high cost and varying size and shape of microcapsules (Burgain et al., 2011).

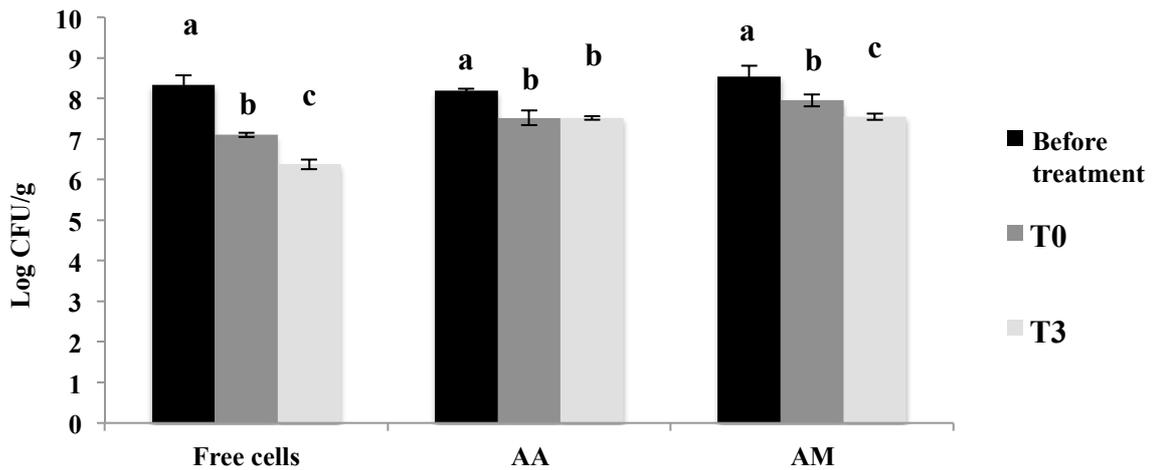


Fig. 3.10 Viable counts of free and microencapsulated *Lactobacillus reuteri* DSM 17938 before and after incubation at 4°C in apricot jam for 0 (T0) and 3 (T3) hours. The error bars represent standard deviations. Different letters labelling bar graph of the same sample category indicate that mean values are significantly different ($p \leq 0.05$) as determined by t-test.

Extrusion, instead, is a simple and cheap method producing homogeneous-shaped capsules with a narrow size distribution, which could be designed for mass production of microcapsules (Whelehan and Marison, 2011). In both systems, due to the gentle operation conditions, a slight damage is usually registered on the bacterial cells (Burgain et al., 2011). On the contrary, spray drying, which is the most commonly used microencapsulation method in the food industry, causes cell envelope damage owing to the high temperature applied during the process (Anal and Singh, 2007).

In this study the vibrating technology, a special system based on the extrusion technique, was applied to microencapsulate cells of the well-known probiotic strain *Lactobacillus reuteri* DSM 17938. Two important outcomes of the microencapsulation process of living cells are the structural quality of the microcapsules and the microencapsulation yield. The former was studied by microscopic investigations and the latter by viable counting of cells released following the disruption of microcapsules. In agreement with Whelehan and Marison (2011) by means of the vibrating technology microcapsules with perfect spherical shape and a diameter of $110 \pm 5 \mu\text{m}$ were obtained (Fig. 3.1). SEM image (Fig. 3.4) showed smaller diameter but it could be a consequence of the preliminary treatment of samples for the microscopic analysis. Solanki et al. (2013) recently reviewed literature on microencapsulation of probiotic cells and reported $356 \mu\text{m}$ as smallest diameter of beads that could be obtained by extrusion technique at that time. On the other hand, microcapsules loaded with fluorescein and produced by vibrating technology at high frequency had a diameter of $158 \mu\text{m}$ although a wide range of other sizes were also reported (Dorati et al., 2013). Our opinion is that the dimension of microcapsules can affect the average number of cells that each microcapsule can hold. Thus a smaller microcapsule containing fewer cells is likely to ensure a more homogeneous behaviour of the bacterial population in it both in terms of resistance to stress conditions and metabolic activity. Furthermore, some authors have alluded to the impact of microcapsule dimension on organoleptic characteristics of some foods. For example, Kailasapathy et al. (2006) found that microspheres of about $300 \mu\text{m}$ containing probiotic bacteria cause a significant increase in the smoothness of yogurt compared to that with free

cells. Similar results were reported by Krasaekoopt and Kitsawad (2010), who found some modifications in the texture characteristics of fruit juice supplemented with microcapsules containing probiotics. Finally, Truelstrup-Hansen et al. (2002) suggested that microcapsules with a size below 100 μm could avoid negative sensory impact in food products.

In this study, the morphology of microcapsules by SEM analysis was investigated and resulting findings indicates that the conditions of microencapsulation applied (i.e. technique, alginate concentration and CaCl_2 solutions, combination of alginate with bacterial cells) promote formation of capsules with reduced diameter, perfectly spherical and continuous in surface dimension. SEM images (Fig. 3.3-3.6) show that cells are not visible on the surface of microcapsules. Furthermore, the absence of cracks ensures the protection of bacterial cells from adverse environment conditions. Also atypical microcapsules (Fig. 3.3b) did not show the presence of cells on the surface, sustaining the hypothesis that they take origin from “collapsing and not breaking phenomena”. Our results on the effect of chitosan coating are also in agreement with those reported by Lee et al. (2004).

The results from this study on the encapsulation yield (Tab. 1) are in agreement with the current literature describing the application of extrusion technique for living cells microencapsulation. Shi et al. (2013) reported an encapsulation yield close to 100% when cells of *Lactobacillus bulgaricus* were microencapsulated in alginate-milk microspheres through vibrating technology. Graff et al. (2007) reported an encapsulation rate of 53% using the same technology to encapsulate *Saccharomyces boulardii* in chitosan-alginate. We report a parameter that is not usually calculated by other authors, that is the estimated number of CFU for each microcapsule (N). In fact, we reasonably assumed that 1 bacterial colony originates from 1 microcapsule, so by counting cells from intact microcapsule samples we produced data for the number of microcapsules. We registered a little difference of N value between AA and AM (Tab. 1), probably due to a release of cells during chitosan coating. Also Graff et al. (2007) did not register reduction of cell load after chitosan coating, while in a previous work (Malmo et al., 2013) we reported a more marked reduction of cell load when chitosan coating was carried out on microcapsules obtained by spray drying. However, our opinion is that spray drying produces microcapsules with features too much different form that obtained by vibrating technology to permit a suitable comparison.

Freeze-drying of microcapsules was performed to confer further viable cell stabilization. Solanki et al. (2013) reported that combining encapsulation with freeze-drying could reduce cell membrane damage from freezing. Our results showed that in addition to having high survival (Tab. 1), microcapsules maintained their morphological characteristics after lyophilization (Fig. 3.5 and 3.6). High cell survival was reported also by Chen and Mustapha (2012), who lyophilized *Lactobacillus acidophilus* microcapsules produced by extrusion/emulsion technique.

Our results showed that cells of *Lactobacillus reuteri* DSM 17938 were well preserved during storage at 4°C when microencapsulated in alginate matrix (Fig. 3.7). However, better probiotic protection systems were described by other authors (Rodrigues et al., 2012; Shi et al., 2013), whose results could be related to the efficiency of a double layer system in preserving the viability of different probiotic strains during a long-term storage. This provides credence to findings from this study, which shows that greater stability could be obtained by coating alginate microcapsules with chitosan or other different matrices. On the other hand, it should be highlighted that different results could be due to different resistance of the microencapsulated strains.

In order to extrapolate their benefit effects on the human or animal hosts, probiotic strains have to resist stressful conditions in the gastro-intestinal tract (Expert Committee FAO/WHO, 2002). *Lactobacillus reuteri* DSM 17938 showed to be very sensitive in gastric-simulated condition (Fig. 3.8). This was also reported in a previous study (Malmo et al., 2013). The results of the current study strengthen the findings from other studies which show that microencapsulation in alginate matrix improve cell resistance to gastric pH, which is further increased when alginate microcapsules are coated with other matrices (Li et al., 2010; Rajam et al., 2012). In contrast with the previous work (Malmo et al., 2013) the microcapsules obtained with alginate alone (AA) did not show any evidence of the effect of bile salts (Fig. 3.8). Again, the difference in findings between this study and the work of Malmo et al. (2013) could be attributed to differences in microencapsulation technologies applied in the different study. Although with little difference, it was observed that AA performed better than AM in protecting probiotic cells from bile salts. This finding is in agreement with results reported earlier by other authors (Li et al., 2010; Malmo et al., 2013). The better protective effect of the AM could be due to the combination of the physical barrier offered by encapsulating matrix and the reaction between chitosan and bile salts that resulted in the formation of an insoluble complex (green stained debris in Fig. 3.9d) and in the limited diffusion of bile salts into the matrix core (Corona-Hernandez et al., 2013).

During food processing and storage, probiotic bacteria if added to the food matrix inevitably get exposed to several stressful conditions (oxidative stress, temperature, pH variations and osmotic stress), which ultimately affect their survival. In considering the effects of the aforementioned stress factors, it is important to recognise the requirement that any probiotic food product must contain at least 10^6 - 10^7 CFU/g of the viable probiotic bacteria at the time of consumption in order to have beneficial effects of the probiotic exerted on human health (Expert Committee FAO/WHO, 2002). High sugar content as found in foods such as confectionaries, jams, fruit nectars, etc., confers high osmotic condition likely to affect survival of probiotic strains in such foods. In this study therefore, we tested the survival of free and microencapsulated cells in apricot jam. Apricot jam was selected to represent foods with high osmotic pressure. Surprisingly, AA exhibited a higher efficacy than AM in protecting probiotic cells from osmotic stress in apricot jam. . The low performance of AM observed could be attributed to the mechanical injury on probiotic cells brought about by increased pressure within microcapsules due to the stronger alginate-chitosan wall following the efflux of alginate interstitial water into the jam matrix.

3.5 Conclusions

This study demonstrated that microencapsulation by vibrating technology can produce stable probiotic microcapsules with dimension and morphological characteristics suitable for food-based applications. In particular, we demonstrated that: (i) alginate-based microcapsules improved *Lactobacillus reuteri* DSM 17938 survival during storage and exposure to gastrointestinal and osmotic stress conditions and that immobilisation of the organism in the alginate-based microcapsules did not affect reuterin production and diffusion of the antimicrobial substance out of the microcapsules; (ii) Co-encapsulation of the probiotic in chitosan-alginate matrix enhanced survival of the probiotic. These findings indicate that microencapsulation by vibrating technology is a promising tool that can be exploited for

protection of probiotic bacteria during food processing, storage and their delivery in foods not usually considered as a vehicle of probiotics.

Notes

This chapter reports the content of original paper entitled “Microencapsulation by vibrating technology of the probiotic strain *Lactobacillus reuteri* DSM 17938 to enhance its survival in foods and in gastrointestinal environment” by De Prisco A. Maresca D., Ongeng D. and Mauriello G., (2015) published on LWT-Food Science and Technology. 61: 452-462.

CHAPTER 4

Enhancement of probiotic stability via entrapment in alginate-based microcapsules during exposure to high temperature

4.1 Introduction

The last decade has encountered an increased interest of researchers towards the encapsulation of probiotic bacteria. The main reason is undoubtedly the enhanced resistance of encapsulated microorganisms to gastrointestinal transit. However, the interest of industry towards the probiotication of different food categories has introduced a new challenge with the respect to the resistance of probiotics to technological hurdles (De Prisco and Mauriello, 2016). Typical technological hurdles experienced in the food industry include low water activity, acidic pH, high salt/sugar content and food additives, high temperature is undoubtedly one of the most challenging to overcome. Indeed, since probiotic strains applied in foods belong mostly to lactic acid bacteria (LAB) group, they are naturally selected following exposure to intrinsic stress factors (e.g. low pH, endogenous microflora, low a_w) associated with specific harsh environment such as those encountered in cheese or sour dough (Corsetti and Settanni, 2007, Shiby and Mishra, 2014). High temperatures applied during food pasteurization, cooking and warming act as barrier for bacteria viability and in case of probiotics, strongly limit their application in baked foods as well as in that foods to be warmed prior to their consuming. Microencapsulation has been proposed as tool to increase cell tolerance to high temperature. Furthermore, its application combined to other technological approach such as the use of prebiotic edible films (Soukoulis et al., 2014a), the addition of cysteine for encapsulation of *Bifidobacteria*, the encapsulation of biofilm-forming bacteria (Cheow et al., 2013, 2014) and the inclusion of microcapsule in starch-based film (Altamirano-Fortoul et al., 2012), frozen bakery products (Malmo et al., 2013) and cream filled cake (Zanjani et al., 2012) have been successfully explored. In the wide scenario of microencapsulation applied for probiotic endurance, most of published works make use, in order of number, needle system, emulsion and spray drying as encapsulation technology. Furthermore, the principal problems of these technologies are that microcapsules are irregular in shape (spray drying), irregular in size (emulsion) and too big to keep food sensorial properties unaffected (needle system). Vibrating nozzle technology, one of the latest developed encapsulation technology based on extrusion principle combine together the production of regular shaped microcapsules with uniform and mouldable sizing. Furthermore, through the application of this technology is also possible to produce two morphological types of microcapsules know as matrix and core-shell microcapsules. On the other hand, it is well known that alginate represents the ideal encapsulating agent due to its low cost, high availability, ability to form strong gel and affinity with living cells. Yet despite, it might result ineffective in protecting bacteria against high temperature or acidic environment due to the too porous structure of the gel. To limit this drawback and to aid the forming of stable and

“insulated” microcapsules it is possible to i) increase the concentration of alginate solution; ii) use co-encapsulating agents; iii) provide a coating to alginate capsules with chemically compatible coating agents and iv) increase the number of layers.

The major challenge of this set of experiments is to conciliate the production of microcapsules able to confer protection toward cells during heat exposure and that result suitable in their size for food application. For this purpose vibrating technology has been applied to produce both matrix and core-shell capsules by using alginate as primary encapsulation matrix, coupled with two natural filling, stabilising and thickening agents as xanthan and locust bean gum, and chitosan used as coating agent for the enhancement of *Lactobacillus reuteri* DSM 17938 viability under thermal stress condition. Finally, bacterial thermoresistance has been assessed in whole fat and skimmed milk in order to evaluate the combined effect of the encapsulation and of different food matrices.

4.2 Materials and methods

4.2.1 Strains and culturing conditions

Lactobacillus reuteri DSM 17938 isolated from Reuterin™ (Noos S.r.l.; BioGaia AB, Stockholm, Sweden) was used. The probiotic strain was cultured in MRS Broth (Oxoid) at 37°C, checked for purity and maintained on MRS Agar (Oxoid). Free and microencapsulated cells of *Lactobacillus reuteri* DSM 17938 were routinely counted on MRS Agar at 37°C for 48 h in aerobic conditions.

4.2.2 Encapsulating and coating solutions

Alginate (Sigma, A2033), xanthan gum (Sigma, G1253) and locust bean gum (Sigma, G0753) were utilized as matrices for microcapsules production. Chitosan (Sigma) was applied as microcapsules coating agent. Alginate solution was prepared by dissolving alginate in preheated deionized water to obtain 1.6 or 1.8% alginate solutions. To prepare alginate-xanthan gum or locust bean gum mixed solutions, both xanthan and locust bean gum were added to 1.6% alginate solution until its complete solubilisation was achieved in order to obtain the highest concentration as possible. Resulting alginate-xanthan and alginate-locust bean gum solution had a final concentration of 2% of solute content with an alginate: xanthan or alginate: locust bean gum ratio of 4:1. Chitosan (Sigma, 448877) solution was prepared by dissolving 0.8% chitosan in preheated (55°C) acetic acid solution (pH of 3.2) as reported by De Prisco et al. (2015). All the solutions were autoclaved immediately after their preparation and left to cool down at room temperature before their use.

4.2.3 Microencapsulation of *Lactobacillus reuteri* cells

Microencapsulation of *Lactobacillus reuteri* cells was performed via vibrating nozzle technology as described in previous works (De Prisco et al., 2015). Alginate-locust bean gum solution was not compatible with extrusion through the 80 µm nozzle. Thus, only alginate and alginate-xanthan solutions were utilized to produce matrix and core-shell microcapsules for a total of four microcapsule systems. Cultures of *Lactobacillus reuteri* in the early stationary phase were harvested by centrifugation at 5200 g for 15 min. Cell pellets were washed twice in sterile quarter-strength Ringer solution (Ringer, Oxoid) and harvested again by centrifugation to be

finally suspended in a volume of 1.6% alginate or alginate-xanthan solution that was equal to the initial volume of cell cultures to obtain a final cell load of 9.18 ± 0.31 . Matrix microcapsules were prepared by extruding cell suspensions through a single-nozzle system having a diameter of 80 μm following procedure by De Prisco et al. (2015). Differently, for core-shell microcapsule production a double concentric-nozzle system was used. The inner nozzle measuring 80 μm in diameter was used for extrusion of cell suspension to obtain the cell-loading core of the capsules. The outer nozzle having a diameter of 200 μm was used for the extrusion of 1.8% alginate solution in order to produce the cell-free shell of microcapsules. Optimal values of membrane vibration frequency, flow rate and of voltage of magnetic field were reported in Tab. 4.1. Hardening of droplets after extrusion was obtained by their incubation in a 0.5 M CaCl_2 solution for 20 min. After the removing of CaCl_2 solution, all microcapsules pellets were washed twice with Ringer; then, they were further coated with a 0.8% chitosan solution by gentle stirring at 200 rpm for 30 min to obtain chitosan coated alginate matrix (AM), alginate-xanthan matrix (XM), alginate core-shell (CAM) and alginate-xanthan core-shell (CXM) microcapsules. Microcapsules were let to sediment, rinsed twice with Ringer solution and finally resuspended in the double of their volume (v/W) of Ringer to be stored at 4°C until their use. An overnight culture of *Lb. reuteri*, prepared in the same day of bacterial cultures used for encapsulation were pelletized, washed twice and finally suspended in a volume of Ringer to be stored at 4°C to be used as control in further tests as following described.

Tab. 4.1 Working parameters in the production of chitosan coated alginate (AM) and alginate-xanthan (XM) matrix microcapsules and chitosan coated alginate (CAM) and alginate-xanthan (CXM) core-shell microcapsules filled with *Lactobacillus reuteri* cells

Parameter	Microcapsules type			
	Matrix		Core-shell	
	AM	XM	CAM	CXM
Nozzle core (μm)	80	80	80	80
Nozzle shell (μm)	-	-	200	200
Flow rate core (ml/min)	3.90	3.90	2.40	2.40
Frequency (Hz)	2600	2900	4300	4300
Electrode (W)	900	1150	2500	2500
Flow rate shell (bar)	-	-	300	300

4.2.4 Enumeration of microencapsulated cells and assessment of microcapsules morphology

Bacterial load of AM, XM, CAM and CXM were calculated on microcapsules in intact form and after their disruption carried out in order to promote the total release of cells from alginate network. Briefly, for intact microcapsules, 2 ml of matrix microcapsules (AM and XM) and 2 g of core-shell microcapsules (CAM and CXM) were decimally serially diluted in Ringer solution while for disrupted microcapsules decimal serial dilutions were performed in 0.2 M sodium citrate (pH 8.0) solution able to destabilize calcium-alginate network. Dilutions were further plate counted on MRS agar as previously described. All microcapsule types were

analysed by using a Zeiss Axiovert 135 light microscope (Carl Zeiss S.p.A., Arese, MI, Italy) equipped with a calibrated micrometer at x 400 and x 100 magnification. About 20 microcapsules from each encapsulation trial were randomly analysed.

4.2.5 *Viability of free and encapsulated Lb. reuteri cells during storage of alginate and alginate-xanthan core-shell microcapsules*

Chitosan coated alginate and alginate-xanthan microcapsules (AM, XM, CAM and CXM) diluted in double of Ringer were stored for 28 days at 4°C and cell viability was weakly checked. At each time-point, microcapsules were disrupted as previously described for determination of total cell load. Free cells were managed in the same way and used as control sample.

4.2.6 *Thermal exposure of free and encapsulated Lb. reuteri cells*

Free and encapsulated *Lb. reuteri* cells were heat treated in hot-water bath (SWB-20, Major Science) equipped with stirring systems to carry out homogeneous thermal treatments and avoid microcapsule sedimentation. In details, 5 ml of AM, XM or 5 g of CAM and CXM and 5 ml of free cells in Ringer were resuspended in ratio 1:9 in preheated whole or skimmed milk and treated for 3, 5 and 10 min at 65 ± 1 and 70 ± 1 °C. Temperature was always checked at the starting and final point of each experiment. Viable counts at 0, 3, 5 and 10 min were performed on microcapsules in disrupted form. Plating was performed on MRS Agar with incubation at 37°C for 48 h in aerobic conditions. Free cells were managed in the same way and used as control sample. Whole and skimmed milk (same brand) were purchased from local market.

4.2.7 *Monitoring of cell damage by a fluorescence microscopy test*

Free and microencapsulated *Lactobacillus reuteri* DSM 17938 cells were stained by using the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes, Eugene, Oregon, USA) to investigate cell membrane damage at different thermal stress conditions. Samples were stained and visualized by epifluorescence microscope according to the procedure followed by De Prisco et al. (2016). Red, orange and green cells indicated damaged, partially damaged and non damaged cells, respectively. Images were captured by a Nikon Coolpix 4500 Digital Camera equipped with a microscope adapter.

4.2.8 *Statistical analysis*

Three independent productions of AM, XM, CAM and CXM were carried out. Further experiments were carried out in duplicate. A t-test analysis (Microsoft Excel for Mac version 11.5) was performed to ascertain significant differences between averages; significance was declared at $P < 0.05$.

4.3 Results

4.3.1 Microcapsules morphology and cell load

Morphology of AM and XM microcapsules are presented in Fig. 4.1 panel a and b, respectively. Chitosan-alginate matrix microcapsules had an average diameter of $110 \mu\text{m} \pm 10$ and a uniform and continuous surface. Bacterial load on intact and disrupted AM microcapsules was of 7.10 ± 0.29 and 9.20 ± 0.25 CFU/ml, respectively. Addition of xanthan to alginate-based capsules led to an increase of the average diameter of XM microcapsules that measured $120 \mu\text{m} \pm 20$ (Fig. 4.1b) and appeared less smooth in their surface than AM capsules with a rougher border-line.

Alginate-xanthan microcapsules had a cell load of 7.00 ± 15 and 8.85 ± 15 CFU/ml, respectively. Both alginate (CAM) and alginate-xanthan (CXM) core-shell (Fig. 4.2a, b) microcapsules showed a similar diameter measuring on $320 \pm 15 \mu\text{m}$ and they appeared perfectly round shaped with uniform border.

Addition of xanthan in let to slight modification in the cell core of the capsules; indeed, as showed in panel b of fig. 4.2 the border-line of cell alginate-xanthan core is less defined than the alginate core in CAM capsule (Fig. 4.2 a) and, as a consequence, a focus CXM capsules showed the presence of few cells in the alginate shell (little square in Fig 4.2 b). Core-shell microcapsules had a similar bacterial load; it was of 5.60 ± 20 CFU/ml and 8.70 ± 23 CFU/ml for intact and disrupted CAM, respectively while CXM carried 5.90 ± 25 CFU/ml and 8.60 ± 15 CFU/ml when in intact and disrupted form, respectively.

4.3.2 Survival of probiotic *Lb. reuteri* cells during storage of alginate and alginate-xanthan core-shell microcapsules

Survival kinetics of free and encapsulated probiotic cells are reported in Fig 4.3. Both encapsulated population in CAM and CXM exhibited a significantly ($P < 0.05$) higher viability than free cells. In details, in *Lb. reuteri* population stored in free form, a total reduction of 3.15 ± 10 log CFU/ml was attested. Differently, slighter reduction (about 2.40 log CFU/g) was observed in *Lb. reuteri* cells in CAM. CXM was the best capsule system in protecting bacteria since a reduction of only 0.90 ± 10 log CFU/g was recorded in *Lb. reuteri* population.

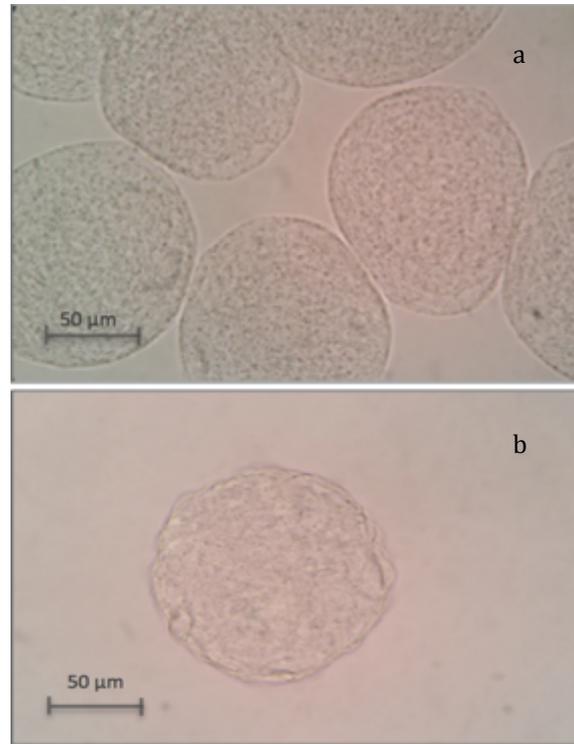


Fig 4.1 Optical images of chitosan coated alginate, AM (a) and alginate-xanthan, XM (b) matrix microcapsules at x 40 magnification

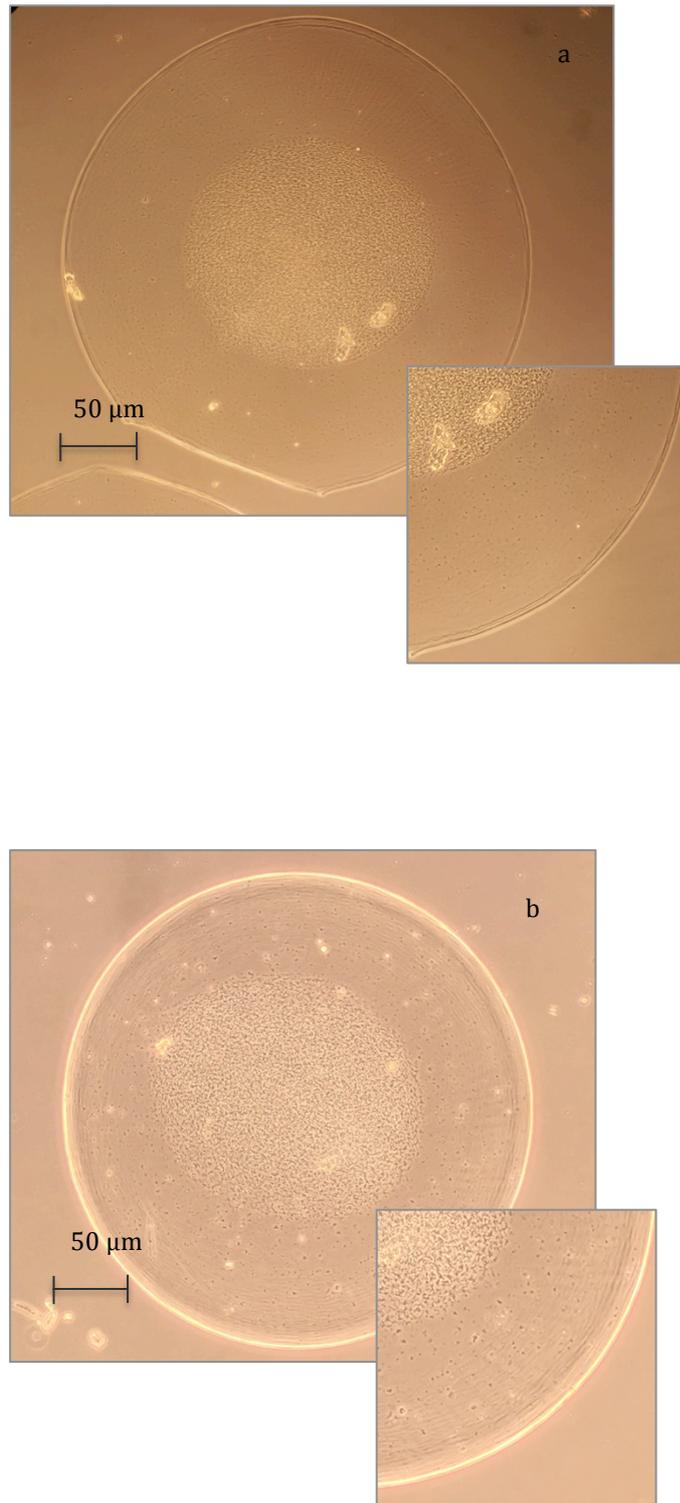


Fig 4.2 Optical images of chitosan coated alginate, CAM (a) and alginate xanthan, CXM (b) core-shell microcapsules at x 10 magnification.

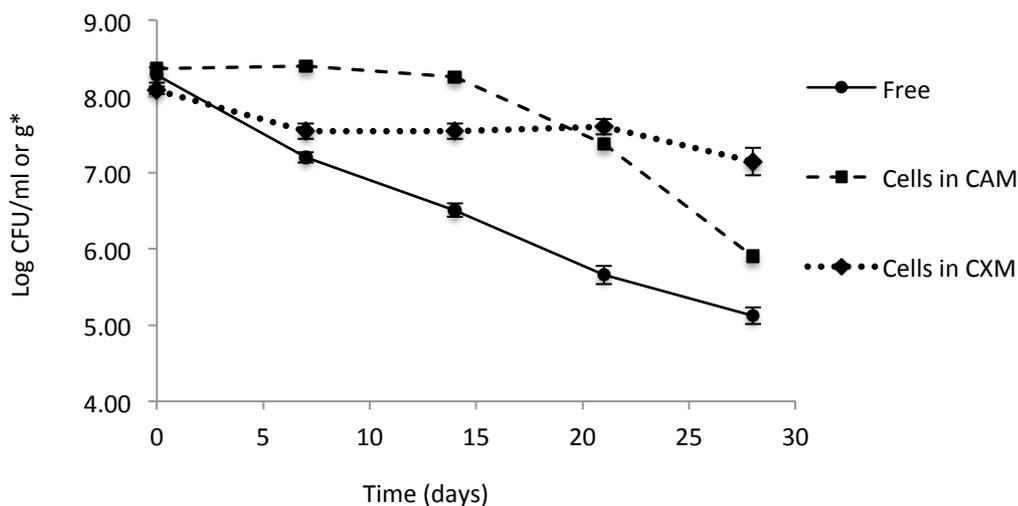


Fig. 4.3 Survival kinetics of free and microencapsulated *Lactobacillus reuteri* DSM 17938 in chitosan coated alginate (CAM) and alginate-xanthan core-shell microcapsules (CXM) during 28 days in Ringer at 4°C. Differences between mean values were attested by *t-test*. Significance was declared at $P < 0.05$. The error bars represent standard deviations. * Bacterial load were expressed in Log CFU/ml for free cells suspension in Ringer and in Log CFU/g for encapsulated cells

4.3.3 Survival of probiotic *Lb. reuteri* cells during high temperature exposure

The effect of microencapsulation on the survival of *Lb. reuteri* exposed to thermal stress conditions (65 and 70°C for 3, 5, and 10 min) in whole and skimmed milk is presented in Fig 4.4 and Fig. 4.5. In almost all the conditions tested, encapsulated bacteria exhibited the higher thermo-tolerance that was always more pronounced in core-shell microcapsule system. Furthermore, among matrix microcapsules, the one made by only alginate (AM) showed the best performance of probiotic cell protection. When threatened at 65 and 70°C in whole milk free cells underwent an averaged reduction of 5.10 and 7.30 log CFU/ml, respectively (Fig 4.4a, b). Encapsulated bacteria survived better in these conditions with a single exception for cells in AM capsules at 65°C that revealed a significantly higher ($P < 0.05$) overall reduction of 6.30 log CFU/ml with respect to free cells. Only increasing milk temperature upon 70°C, an effective advantage of AM microcapsules was revealed as demonstrated by a reduction of 5.70 ± 10 Log cycles for encapsulated cells versus the reduction 7.30 log CFU/ml of free cells. However, the inclusion of xanthan gum to alginate improved the protective effect of matrix capsules in all the conditions tested. Among core shell microcapsules, CAM showed the best performance of bacteria protection as indicated by cell reduction of only 1.30 and 4.10 log CFU/g in whole and skimmed milk, respectively. Incubation in skimmed milk during thermal treatment was more markedly bacterial viability in comparison whole milk as reported in (Fig. 4.5 a, b) and as depicted in Fig. 4.6 (a and b). Even in skimmed milk encapsulated population faced better the stress from high temperature than the free counterparts (Fig. 4.5 a, b) and strongest resistance was observed in cells in core shell microcapsules. In details, no significant ($P < 0.05$) differences from treatment in whole milk were evidenced in the viability of free cells and cells in CXM capsules when treated in skimmed milk at 65°C. Differently a higher resistance was observed in cells in CAM capsules in skimmed milk with respect to the same treatment in whole milk, since an averaged reduction of 5.20 log CFU/ml was recorded

(versus a reduction 6.80 log CFU/ml in whole milk). Treatment at 70°C in skimmed milk was completely detrimental for free bacteria that showed no survival after 5 min of treatment.

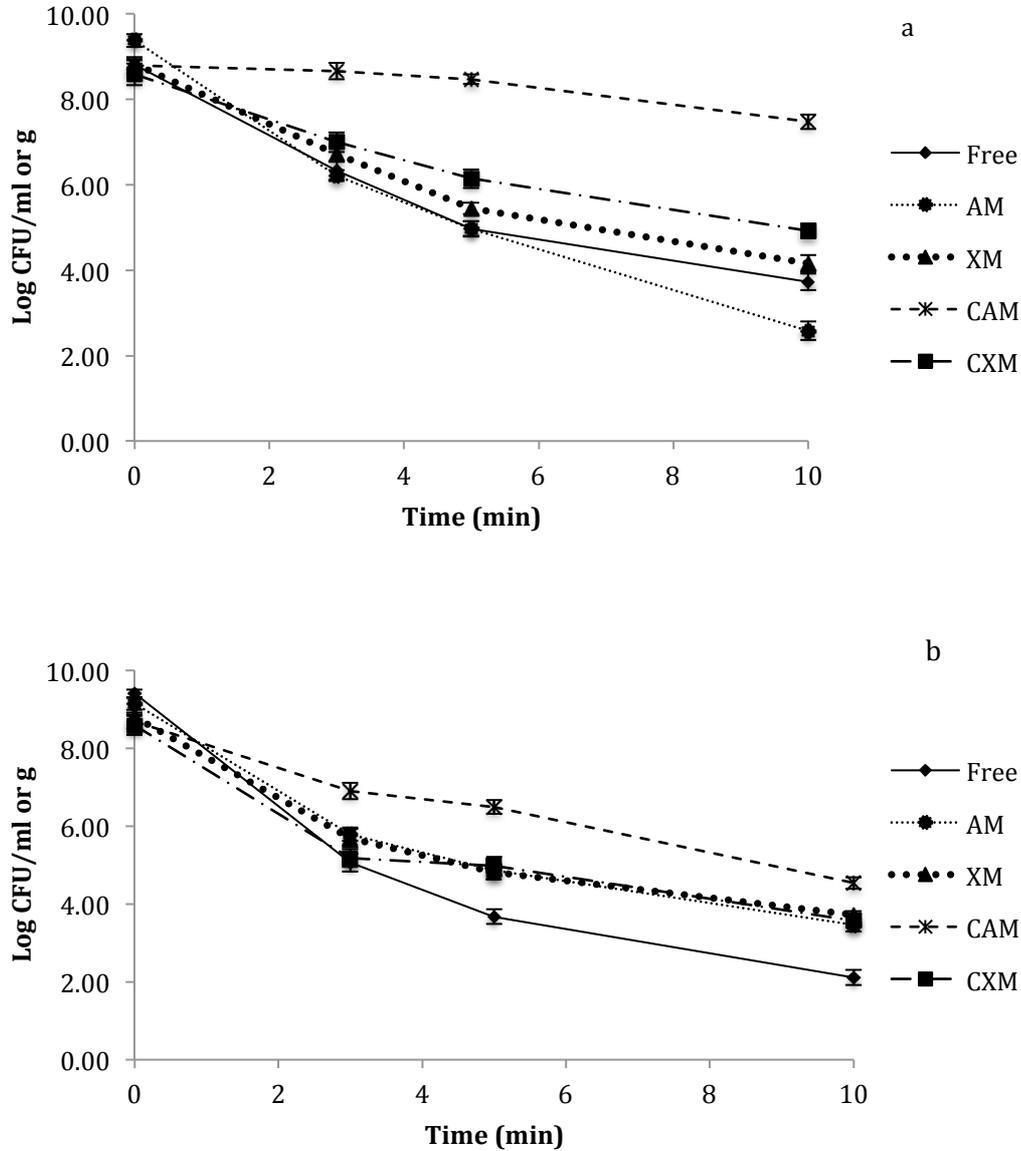


Fig. 4.4 Survival kinetics of free and microencapsulated *Lactobacillus reuteri* DSM 17938 in chitosan coated alginate (AM) and alginate-xanthan (XM) matrix capsules and in chitosan coated alginate (CAM) and alginate-xanthan (CXM) core-shell microcapsules during exposure at 65 (a) and 70°C (b) for 3, 5, and 10 min. in whole fat milk.

Differences between mean values were attested by *t*-test. Significance was declared at $P < 0.05$. The error bars represent standard deviations. * Bacterial load were expressed in Log CFU/ml for free cells suspension in Ringer and bacterial population in matrix type system and in Log CFU/g for bacterial population in core-shell microcapsules

Differently, a clear effect of protection of all encapsulating systems was appreciable in Fig 4.5b where the best performances were observed for *Lb. reuteri* cells in CAM microcapsules with a reduction after 10 min at 70°C of about 4.30 log CFU/g.

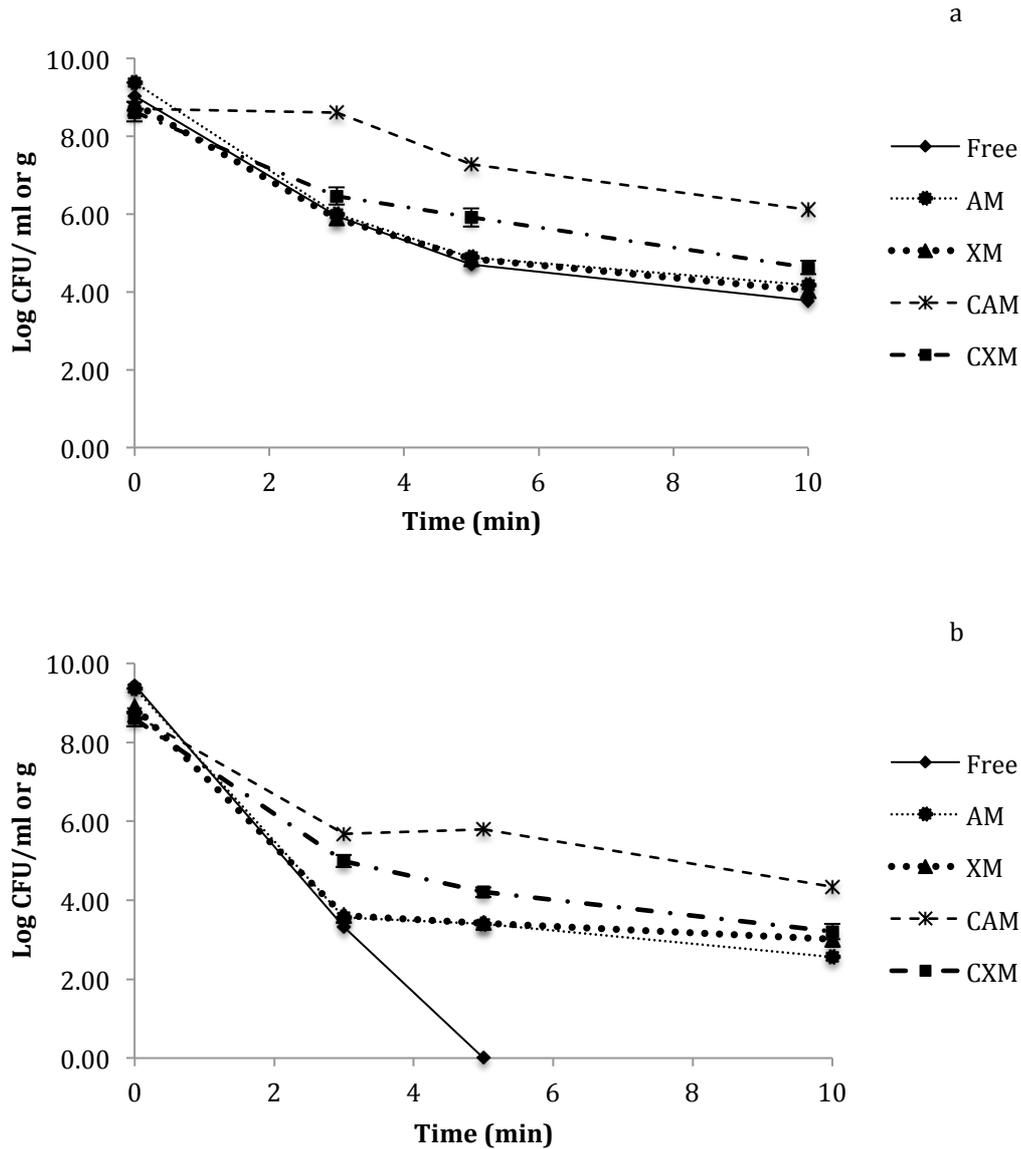


Fig. 4.5 Survival kinetics of free and microencapsulated *Lactobacillus reuteri* DSM 17938 in chitosan coated alginate (AM) and alginate-xanthan (XM) matrix capsules and in chitosan coated alginate (CAM) and alginate-xanthan (CXM) core-shell microcapsules during exposure at 65 (a) and 70°C (b) for 3, 5, and 10 min. in skimmed milk.

Differences between mean values were attested by *t-test*. Significance was declared at $P < 0.05$. The error bars represent standard deviations. * Bacterial load were expressed in Log CFU/ml for free cells suspension in Ringer and bacterial population in matrix type system and in Log CFU/g for bacterial population in core-shell microcapsules

4.3.4 Probiotic *Lb. reuteri* cell damages during high temperature exposure

For all microcapsules, the prevalence of orange/red cells gradually increased with time of exposure to high temperature. Cells in A and X microcapsules at 70°C in whole milk fat are depicted in Fig 4.6a, b. Prevalence of red stained cells is appreciable both in A and in X capsules even though during the same treatment their incidence seems to be higher in X microcapsules (panel b). Fig. II and III of panel b clearly indicate the gradual brake down of the alginate-xanthan gel network, suggesting the reason of the higher protective performances

of A capsules. Interestingly, through this staining technique it was evidenced a not uniform red staining in encapsulated cell population indicating a likely stronger gel network in correspondence of green cells that resulted more protected.

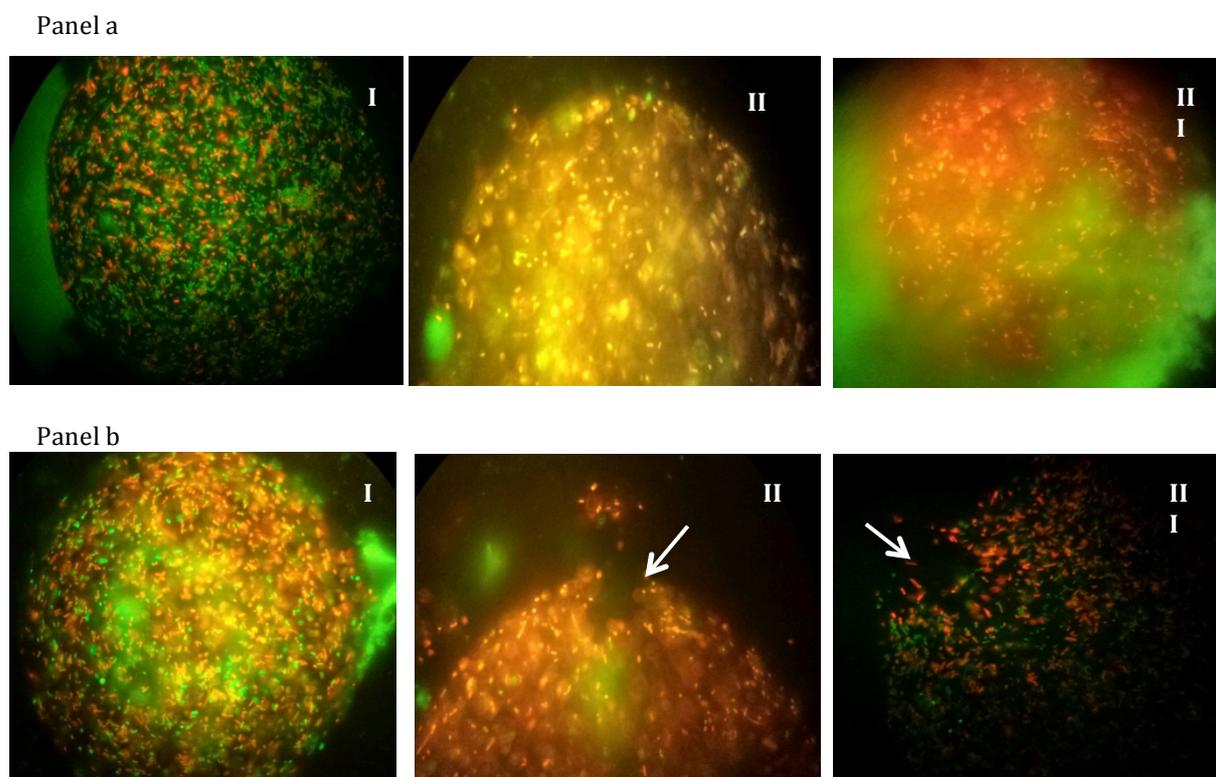


Fig. 4.6 Fluorescence microscopy images at x 40 magnification of stained *Lactobacillus reuteri* DSM 17938 in chitosan alginate (panel a) and chitosan-alginate-xanthan matrix microcapsules (panel b) during thermal exposure at 70°C in whole fat milk for 3 (I), 5 (II) and 10 (III) min.

4.4 Discussion

The application of encapsulation as tool for the enhancement of bacterial thermoresistance is a very attractive and potential area that is gaining researcher interest, as documented by the increasing number of papers available on this topic (Fritzen-Freire et al., 2013; Endo et al., 2014). Along with it, the production of microcapsules suitable for food application in terms of dimension is also a fundamental challenge to avoid sensorial drawbacks.

With these experiments we aimed to test the protective performance at high temperatures of probiotic chitosan-alginate based microcapsules, modified in their composition, for the addition of xanthan gum, and in their structure since core-shell capsules have been obtained with the smallest diameter as possible for vibrating technology.

The four types of microcapsules (AM, XM, CAM and CXM) obtained have also been characterized for cell loading, morphology and cell protection ability during refrigerated storage. Bacterial load was determined on microcapsules in intact and disrupted form with the main aim of counting the whole encapsulated bacterial population. At this purpose, differently from our previous work (De Prisco et al., 2015) where we reported the use of phosphate buffer for capsules disintegration, we used a solution of 0.2 M sodium citrate pH 8.00 since it was more efficacious in dissolving alginate network also in dairy matrices. All microcapsules

showed a high bacterial load, and this is in line with our previous experiments indicating the high cell capacity load of alginate microcapsules obtained by vibrating technology and a high encapsulation efficiency approaching the 100% (De Prisco et al., 2015). For the first time we obtained, by a double extrusion through a concentric nozzle system, perfectly round shaped core-shell microcapsules with an averaged diameter of about 320 μm and a perfect distribution of cell-core and shell compartments. Due to the increased size of core-shell capsules with respect to matrix capsules (about 120 μm) and resulting difficult handling of them, counting of core-shell capsules has always been performed on 1 gr. Indeed, preliminary results indicated high standard deviations in counting performed on 1 ml (data not showed). Most likely, this phenomenon was own to rapid capsule sedimentation during routinely operations and different number of microcapsules analysed at each experiment per ml. Filling alginate microcapsules with xanthan gum led to a slight increase in the capsules diameter (Fig. 4.1). Similarly to our results, an increment of alginate microcapsule diameter following the addition of xanthan was also reported by Cheow et al. (2014). Reasonably, this increase in XMC could be attributed to i) the most probable higher viscosity of alginate-xanthan suspension; ii) the swelling capacity of xanthan; iii) the bonds created by xanthan and alginate that could reduce the linkage of calcium ions and the consequent water molecules expulsion from the network. Likewise, this phenomenon implicated what observed in the morphology of CXM, where a weak borderline of cell-core was evidenced as also suggested by some cells visible in the shell (Fig. 4.2). The slower contact of Ca^{2+} ions with alginate-xanthan core due to the primary polymerization of the alginate shell could also explain what evidenced in the morphology of CXM capsules.

Probiotic core-shell microcapsules (CAM and CXM) were stored in Ringer solution at 4°C since in a previous work this storage condition was the best to preserve bacterial viability (De Prisco et al., 2015). CXM explained the higher protective effect on *Lb. reuteri* cells. This type of microcapsule showed to be the most effective in terms of prolongation of bacterial viability over CAM and previously tested AM (De Prisco et al., 2015). Differently, Fareez et al., (2015) reported better results during the storage of *Lactobacillus plantarum* LAB12 encapsulated in alginate only rather than in alginate-xanthan matrix. Thus, we could assume a strain-specific beneficial effect of xanthan.

Data about the enhanced bacterial viability against high thermal stress are hardly comparable due to the different thermal conditions and probiotic strains tested as well as encapsulating matrices and suspending media utilized. Accordingly, it is difficult to establish which, among the different microcapsule systems, is the most effective for this item. Many approaches have been experienced to confer thermoresistance to probiotic cells such as i) the combined use of encapsulation/cell inclusion in edible film (Altamirano Fortoul et al., 2012; Soukoulis et al., 2014); ii) the use of low-water affinity coating agent to reduce heat moist diffusion into the capsules (Mandal et al., 2014; Pitigraison et al., 2017); iii) encapsulation of biofilm forming bacteria to exploit their innate robustness (Cheow and Hadinoto, 2013; Cheow et al., 2014).

Lactobacillus reuteri DSM 17938 did not show in its native form a notable thermoresistance; indeed, other probiotic strains such as *Lb. plantarum* TN8 and *Lb. rhamnosus* GG behave better when tested against high temperatures (Abbaszadeh et al., 2013; Trabelsi et al., 2013). All microcapsules examined in this study were able to increase the survival of *Lactobacillus reuteri* cells during almost all the high temperature conditions tested (Fig 4.4 and 4.5) with greater preservation effect for core-shell morphology. These results confirmed what reported in literature about the higher efficacy of multi-layered microcapsules and of microcapsules

obtained by high-percentage alginate solution (Mandal et al., 2014). Alginate used as primary agent for capsules production showed promising thermo-protective action. This behaviour could be explained by alginate shrinkage that occurred for water expulsion from alginate gel during heat treatment leading to an increased in alginate density and the formation of more resistant and less permeable gel (Serp et al., 2002; Malmo et al., 2013). Interestingly, in accordance with Fareez et al., (2015) xanthan gum improved the preservative effect of matrix microcapsules. On the contrary, this effect was not evidenced in core shell capsules since CAM capsules were more efficacious in enhancing bacterial viability under high temperature. This evidence might be explained by the proper morphology of CAM capsules especially for what concern the core of the capsules that might create a stronger gel around cells when made of only alginate.

Noteworthy are the results obtained by Cheow et al., (2014) and by Pitigraisorn et al., (2017). First authors let *L. rhamnosus* cells grow into alginate-xanthan capsules forming natural biofilm that act as a further natural barrier against temperatures, obtaining a good cell survival at 100°C for 45 min in lyophilized capsules (Cheow et al., 2014). Pitigraisorn et al., (2017) encapsulated *L. acidophilus* cells in multi-layered capsules obtained via electrospraying and fluidized bed coating by alginate, egg albumen, stearic acid and cassava starch granules. When treated at 70°C for 30 min, encapsulated *L. acidophilus* cells only suffered a reduction of 0.6 log. However, microcapsules dimension tested in both studies was of at least 500 µm and the encapsulation process used in the above mentioned works are by far more complex than encapsulation in alginate-based capsules by vibrational nozzle technology.

4.5 Conclusion

Encapsulation is a key strategy to preserve cell viability toward technological stress imposed by food extrinsic and intrinsic factor, storage and consumption. Accordingly, though a simple and non time-consuming encapsulation technique and cheap and safe polymer matrices, we obtained core-shell microcapsules able to increase the thermoresistance of probiotic cells and their viability during storage. Xanthan gum revealed a preservative effect toward cells during storage at 4°C and under thermal stress, even though only when included in matrix morphology. Accordingly, chitosan coated core-shell microcapsules obtained by alginate alone explicated the higher thermo-protective effect toward *Lb. reuteri* cells. Together, the present findings strongly encourage the elucidation of the possible advantageous interaction of xanthan with probiotic *Lb. reuteri*. They also strengthen the possibility of include probiotics encapsulated in natural polymer matrices in non dairy and non dairy product for which mild heat treatment or warming is required. Further experiments could be performed to assess the effect of thermo-protection toward other many probiotic strains.

Notes:

This group of experiments has being finalized for further publication. Additional experiments are ongoing and they included: i) microcapsules lyophilisation; ii) thermal exposure of lyophilized microcapsules; iii) better assessment of the influence of food matrix of microcapsules morphology.

CHAPTER 5

Endurance of new potentially probiotic *Lactobacillus* strains to simulated gastrointestinal conditions through alginate-based microencapsulation

5.1 Introduction

According to current literature, probiotic bacteria are microorganisms able to confer health benefits to the host when administered in adequate amounts in live and active form (Expert Committee FAO/WHO, 2002; Bhathena et al., 2013). On the basis of this premise, probiotics need to overcome detrimental factors encountered in the gastrointestinal (GI) tract so as to retain their viability and important physiology (Lebeer et al., 2008). That of probiotic health benefits is an area of intensive research in different domains that, according to the target sites and mechanism of action, could be distinguished in intestinal and extra-intestinal (De Prisco and Mauriello, 2016). Probiotics are largely consumed for the maintenance and restoring of the normal intestinal microflora (Douglas and Sanders, 2008) but evidences about their potentiality in facilitate lactose metabolism, treatment of infantile diarrhea, reduction of serum cholesterol level and blood pressure and prevention from some cancers are also available (Khani et al., 2012). Accordingly, the reported evidences for the relevant impact of probiotics on diverse end points of human health is driving the commercial development of products containing them (Tripathy & Giri, 2014). However, after the administration of many probiotic strains, they might suffer a considerable loss of viability attributable to the injurious action of human factors (e.g. bile salts, low pH) encountered during the GI transit (Mokarram et al., 2009; Rodriguez et al., 2011; Zhao et al., 2012; Malmo et al., 2013). In this context, microencapsulation of probiotics in different matrices, especially hydrogels, has widely demonstrated to be a suitable strategy to preserve bacterial viability from this harsh environment and to target their release in the intestine (Brun-Graeppi et al., 2009; Cook et al., 2012; Heideback et al., 2012). Over the extensively demonstrated effects of microcapsules to enhance bacteria resistance in simulated GI condition, the domain of the encapsulation as strategy for probiotic human delivery also account successfully applications i) to enhance the resilience time of probiotics encapsulated in alginate/poly-L-lysine/alginate/chitosan to promote the *in situ* production of folate (Ramos et al., 2016); ii) to increase bacterial viability under GI transit after previous inclusion and storage in foods (Picot and Lacroix, 2004; Sandoval-Castilla et al., 2010; Chaikham et al., 2012, 2016) and iii) for probiotic oral delivery in *in vivo* test (Del Piano et al., 2012). However, the wealth of works dealing with this topic is mainly focused on the encapsulation of well-known and commercialized probiotic strains that are expected to hold intrinsic notable features of resistance against GI factors. Differently, the effects of microencapsulation toward strains showing interesting probiotic and technological feature but a weak resistance to the GI tract are not still explored. On this premise, this Chapter reports the investigation of potentially protective activity in GI conditions toward new *Lactobacillus* strains of microcapsules previously tested for their protective performance

under high temperatures. Furthermore, saliva, gastric and intestinal conditions have been simulated, at target pH values, using lysozyme, pepsin and a pool of pancreatic enzymes and bile salts dissolved in saline solutions in order to reproduce as strictly as possible the real human gastrointestinal environment.

5.2 Materials and Methods

5.2.1 *Strains and culture conditions*

New potentially probiotic strains BR35, BR7 and BM4, belonging to *Lactobacillus* group used in the present set of experiments have been previously isolated in our laboratory from faecal samples of healthy breast-fed babies aged between 0 and 3 months. Their belonging to *Lactobacillus* genes and identification at species level has been further accomplished through the sequencing of 16S rRNA tract (Maresca et al., submitted to PLOSone). The new isolated strains selected for this study BR35, BR7 and BM4 were grown in MRS broth (Oxoid) at 37°C in aerobic conditions and cultured on MRS agar at the same conditions for their enumeration.

5.2.2 *Microencapsulation of BR35, BR7 and BM4 isolates*

Cultures of BR35, BR7 and BM4 in their early stationary phase were harvested by centrifugation at 5200 g for 15 min. Cell pellets were washed twice in sterile quarter-strength Ringer solution (Ringer, Oxoid) resuspended and harvested again by centrifugation to be used for encapsulation experiments. Cell pellets were resuspended in 1.6% alginate and 2% alginate-xanthan solution obtaining a cell concentration of 8.15 ± 0.20 Log CFU/ml to be microencapsulated in matrix and core-shell microcapsules. Microencapsulation and chitosan-coating were performed according to the procedure and parameters described in Chapter 4. Definitely, all strains (BR35, BR7 and BM4) were encapsulated in chitosan coated alginate (AM) and alginate-xanthan (XM) matrix microcapsules and in chitosan coated alginate (CAM) and alginate-xanthan (CXM) core-shell microcapsules. After their production, microcapsules rinsed twice with Ringer solution were finally resuspended in a volume of Ringer that was equal to the initial volume of the cell cultures in order to restore the initial cell concentration. Microcapsules were stored at 4°C until their use.

5.2.3 *Enumeration of microencapsulated cells*

Bacterial load of AM, XM, CAM and CXM were calculated on microcapsules in intact and disrupted form in accordance to what described in Chapter 4. Briefly, for intact microcapsules, 2 ml of matrix microcapsules (AM and XM) and 2 g of core-shell microcapsules (CAM and CXM) were decimally serially diluted in Ringer solution while for disrupted microcapsules decimal serial dilutions were performed in 0.2 M sodium citrate (pH 8.0) solution. Samples were further plate counted on MRS agar as previously described and counts of disrupted microcapsules were considered the bacterial load of samples before the GI treatment.

5.2.4 *Cell survival after consecutive passages through simulated saliva, gastric and intestinal solutions*

A model stomach/intestinal passage experiment developed by Vizoso-Pinto et al. (2006) was here adapted to compare the survival of potential probiotic strains in their free and

encapsulated form. To simulate the dilution of bacteria in the human oral cavity, cell pellet from 5 ml of bacterial cultures, 5 ml of matrix microcapsules or 5 g of core-shell microcapsules loading BR35, BR7 or BM4 were diluted 1:1 in a saliva simulating solution (SSS) at pH 7.2 containing an electrolyte solution of 6.2 g/l NaCl, 2.2 g/l KCl, 0.22 g/l CaCl₂ and 1.2 g/l NaHCO₃ and 100 ppm lysozyme and incubated for 5 min at 37°C. The sample was subsequently diluted 1:4 (with respect to the sample initial volume) with a gastric simulated solution (GSS), consisting of the above mentioned electrolyte solution adjusted at pH 2.5 plus 0.3% pepsin. After 2 h of incubation at 37°C samples were further diluted in ratio 1:5 in an intestinal simulating solution (SSS) at pH 7.2 consisting of 6.4 g/l NaHCO₃, 0.239g/l KCl, 1.28g/l NaCl with 0.5% bile salts and 0.1% pancreatin to be incubated for 3 h at 37°C. During gastric and intestinal incubation, samples were manually mixed at interval of 20 min. One ml aliquots of each sample were withdrawn after saliva, saliva plus gastric and saliva plus gastric plus intestinal incubation to be serially diluted in Ringer and counted on MRS agar. Bacterial load of encapsulated samples was determined on disrupted microcapsules. All chemicals were purchased from Sigma Aldrich. Cell membrane state during the treatment in GI condition of free and microencapsulated cells was analysed by using a LIVE/DEAD[®] BacLight™ Bacterial Viability Kit (Molecular Probes, Eugene, Oregon, USA). Samples were stained and visualized by epifluorescence microscope according to the procedure described in Chapter 4.

5.2.5 Statistical analysis

Three independent experiments were carried out in duplicate. One way analysis of variance test (Microsoft Excel for Mac version 11.5) was performed to ascertain significant differences between averages; significance was declared at $P < 0.05$.

5.3 Results

5.3.1 Bacterial load of microcapsules and entrapment efficiency (EE)

The encapsulation in four microcapsule systems, different for morphology and composition, was successfully carried out with all the bacteria tested. None of the strains were negatively affected by the encapsulation by vibrating technology. Bacterial loads estimated on aliquots of 2 ml and 2 g of matrix and core-shell microcapsules, respectively are reported in Table 5.1. By using the method reported in Chapter 3 for the evaluation of the entrapment efficiency (consisting in dividing the viable count of disrupted microcapsules by the cell load before the microencapsulation), the efficiency of microencapsulation was found to approximate the 100% for BR35, BR7 and BM4.

5.3.2 Bacterial survival in gastrointestinal simulated conditions

Three newly isolated and potentially probiotic strains have been enclosed in different microcapsule systems (AM, XM, CAM, CXM) and then treated under simulated gastrointestinal conditions. Their behaviour when treated in free end encapsulated form is reported in Fig. 5.1. All strains (BR35, BR7 and BM4) showed to be fully resistant to the stress from saliva (Lysozyme) simulating solution. Differently, when treated in free form all the strains showed different ability in coping with stress imposed by gastric (low pH and pepsine) and intestinal (bile salts, pancreatic enzymes) harsh conditions. Among them, BR7 showed the

higher survival during the incubation in GSS and the subsequent incubation in ISS with a survival percentage of 18 and 12%, respectively. About the 1 and only the 0.5% of survived cells was instead recovered in free population of BM4 and BR35, respectively, after the complete passage through the GI simulated tract. When encapsulated in matrix microcapsules these potentially probiotic strains benefited of this condition as demonstrated by the higher level of survival in the GI tract but with different extent.

Tab. 5.1 Bacterial load (Log CFU/ml or g) of chitosan coated alginate and alginate-xanthan matrix microcapsules (AM and XM) and chitosan coated alginate and alginate-xanthan core-shell microcapsules (CAM and CXM) obtained by using a cell suspension of BR35, BR7 and BM4 strains of 8.15 ± 0.20 Log CFU/ml

Microcapsules	BR35		BR7		BM4	
	Intact	Disrupted	Intact	Disrupted	Intact	Disrupted
AM	6.00±0.15	8.00±0.10	6.10±0.18	8.00±0.21	6.15±0.20	8.05±0.15
XM	6.44±0.12	8.05±0.23	6.36±0.08	8.10±0.12	6.45±0.15	8.10±0.22
CAM	5.50±0.20	8.10±0.10	5.50±0.20	8.15±0.15	5.50±0.20	8.10±0.20
CXM	5.90±0.19	8.15±0.15	5.90±0.19	8.10±0.11	5.90±0.19	8.20±0.10

* * Results show means ± standard deviation of three independent experiments carried out in duplicate.

Indeed, interestingly, both AM and XM provided different effects on bacterial strains indicating a likely “strain-specific” protective effect of the microencapsulation since, for example, AM saved about 1.2, 1.5 and 1.0 log cycles in BR35, BR7 and BM4 population, respectively. Even though all the strains took advantage from encapsulation in both AM and XM, AM showed a significantly higher level of cell preservation (Fig. 5.1). Furthermore, it has to be highlighted the loss in viability of BR7 and BM4 in SSS when encapsulated XM. The core shell morphology improved the protective action of alginate and alginate-xanthan based microcapsules (Fig. 5.2). The viability of all the strains tested increased up to raise about the 90% of survival at the end of the GI incubation for all the strains encapsulated in CAM and CXM and for two (BR35, BM4) out of the three strain tested CAM explicated the high protective action. An indication about cell integrity and microcapsule morphology along the GI tract is appreciable in Fig. 5.3 that depicts the state of free and encapsulated strain BR7. A gradual red staining, indicating cell membrane damage is appreciable during cell incubation in GSS and ISS. This phenomenon is attenuated by the protective action of chitosan-alginate and alginate-xanthan gel, although the best situation after ISS is the one obtained in AM. Both AM and XM microcapsules retained their morphology but they undergone a partially braking up in ISS, required for cell release into the intestinal compartment.

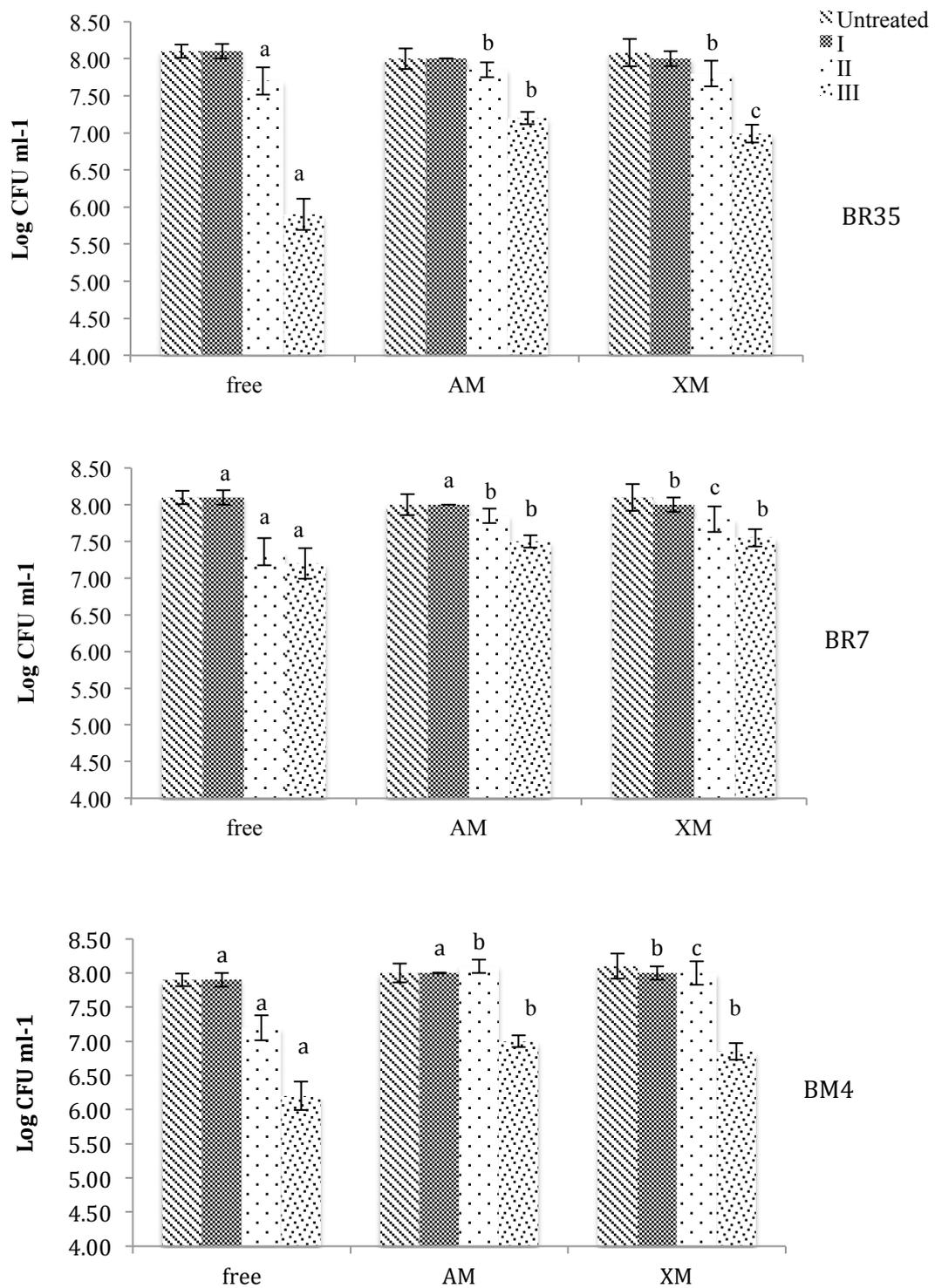


Fig. 5.1 Viable counts of three newly isolated and potentially probiotic *Lactobacillus* strains (BR35, BR7 and BM) tested during subsequent incubation in saliva (I), gastric (II) and intestinal (III) simulating solutions in free form and microencapsulated in chitosan-coated alginate (AM) and chitosan-coated alginate xanthan matrix (XM) capsules. Data are expressed as mean values of three independent replicas of experiment and bars represent standard deviation. Different letters labeling bar graphs of the different sample categories (free, AM and XM) in the same conditions (I, II and III) indicate that mean values are significantly different ($P < 0.05$) as determined by t-test.

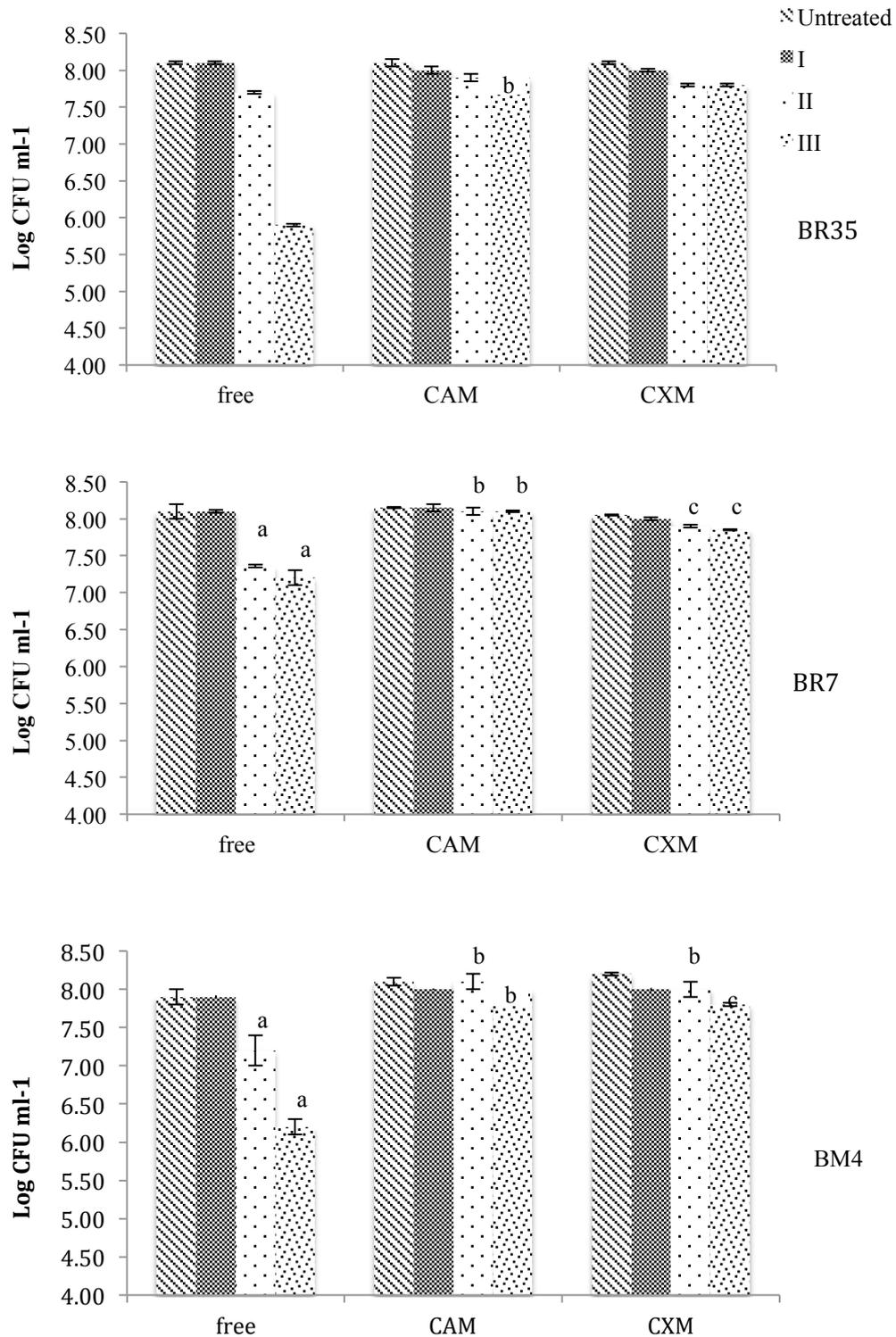


Fig. 5.2 Viable counts of three newly isolated and potentially probiotic *Lactobacillus* strains (BR35, BR7 and BM4) tested during subsequent incubation in saliva (I), gastric (II) and intestinal (III) simulating solutions in free form and microencapsulated in chitosan-coated alginate (CAM) and chitosan-coated alginate xanthan (CXM) core-shell capsules. Data are expressed as mean values of three independent replicas of experiment and the bars represent standard deviation. Letters labeling bar graphs of the different sample categories (free, AM and XM) in the same conditions (I, II and III) indicate that mean values are significantly different ($P < 0.05$) as determined by t-test

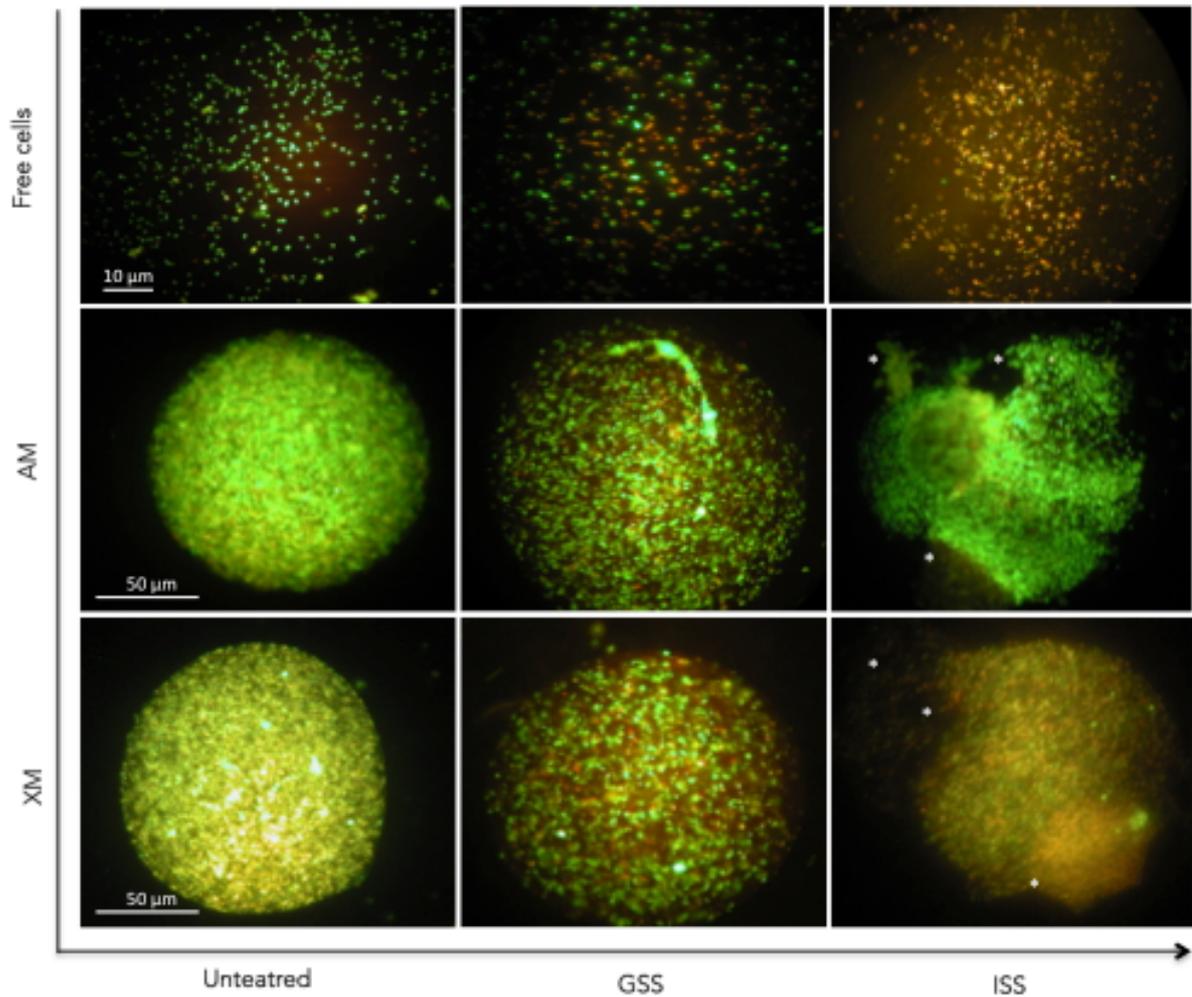


Fig. 5.3 Fluorescence microscopy images of viable staining of free BR7 (X 100 magnification) and encapsulated in chitosan coated alginate (AM) and alginate-xanthan matrix microcapsules ((X 40 magnification) during incubation in gastrointestinal conditions (GSS for 2h and ISS for 2h). White spots indicate the breaking up of the alginate network.

5.4 Discussion

Lactobacillus strains involved in these experiments have been previously obtained during an isolation from human faecal samples aimed at the discovering of new lactobacilli belonging to *Lb. joseri* and *Lb. johnsonii* species. A selected group of about 50 isolates identified as *Lb. gasseri*/*L. johnsonii* was further studied for technological and metabolic properties (i.e. ability to grow aerobic or respiratory conditions, catalase production, toleration of oxidative stress imposed by ROS generators) and for probiotic features such as gastrointestinal resistance and antimicrobial activity (Maresca et al., submitted to PLOSone). Strains BR35, BR7 and BM4 exhibited interesting metabolic features and they were found to explicate, during agar spot and well diffusion agar test, antimicrobial properties against pathogen and spoilage bacteria but, alongside, they also showed poor resistance to the GI conditions. On this base, microencapsulation has been tested as strategy to confer the gastrointestinal resistance to interesting strains natively lacking of this property. In particular, the preservation feature of AM, XM, CAM and CXM toward BR35, BR7 and BM4 has been evaluated during *in vitro* tests

based on the use of lysozyme, pepsin and a pool of pancreatic enzymes and bile salts dissolved in saline solutions adjust to optimal pH for an adequate mimicking of human oro-gastro-intestinal environment. Our preliminary experiments (data not shown) carried out with alginate and chitosan coated alginate microcapsules suggested that the chitosan coating was essential to preserve the intact morphology of alginate capsules during the gastrointestinal incubation performed according to Vizoso-Pinto et al. (2016). For the abovementioned evidence, we included in this study only the chitosan-coated microcapsules.

Findings here reported indicate that all microcapsules exerted a beneficial effect on *Lactobacillus* strains. In the case of matrix microcapsules system, capsules made by alginate and coated with chitosan had a slightly higher protective effect toward all the strains tested. What was observed could be explained with a higher strength in chitosan-Ca²⁺-alginate gel with respect to the same gel added of xanthan. According to what also observed in the previous Chapter of this thesis, it is probable that alginate-xanthan matrix might form a more porous gel structure that make the microcapsules more permeable to the external compounds. This hypothesis should be undoubtedly confirmed with a more sophisticate capsule image and structural analysis. Furthermore, our results clearly indicated that encapsulation in core-shell morphology further improved bacterial resistance (Fig. 5.5). The bigger microcapsules sizing and the additional protective layer of alginate can reasonably explain this finding. Several encapsulating matrices have been screened as carrier to endure probiotics against gastrointestinal harsh condition; alginate coupled with filling agent or coated with poly-L-lysine or chitosan, whey proteins, gelatin and cellulose-acetate-phthalate are some examples (Ding et al., 2009; Nazzaro et al., 2009; Rodriguez et al., 2011; Heidebach et al., 2012; Ramos et al., 2016). Among them, the effectiveness of alginate-chitosan complex in the efficient GI delivery of probiotic bacteria has been widely and successfully explored due to its gastric resistance characteristic and its capacity to release cells at pH >7.0 (Anal and Singh, 2007; Dong et al., 2013; D’Orazio et al., 2015). Our results about the behavior of chitosan-alginate capsules obtained by vibrating technology confirmed this evidence. This was already demonstrated in our previous work (De Prisco et al., 2015), however, this experiment is based on the use of a more complex protocol for the *in vitro* evaluation of cell GI resistance in which the different chemical conditions can affect both cell viability and microcapsules behavior. Alginate based microcapsules obtained by vibrating technology showed good properties during GI delivery of BR35, BR7 and BM4 (Fig. 5.1 and 5.3). This is a promising findings since, beside the encapsulation matrices, the technology applied for microcapsule fabrication can strictly affect their structural properties and resulting functional performances as also demonstrated by Musikasang et al. (2009) who found that alginate based capsules produced by extrusion better protect lactic acid bacteria then the counterpart obtained by emulsion. A strain-dependent behaviour in taking advantage from microencapsulation procedure was observed and this is in line with previous findings about the performance of different species of probiotic lactic acid bacteria (Borges et al., 2012).

5.5 Conclusion

Cell loaded alginate-based capsules obtained by vibrating technology were exposed to gastrointestinal simulated passage in order to evaluate their ability to protect three new isolated and potentially probiotic *Lactobacillus johnsonii/gasseri* strains from this harsh environment. All microcapsules (AM, XM, CAM and CXM), irrespective of differences in

morphology (matrix and core-shell) and composition (alginate and alginate-xanthan mix) improved the gastrointestinal resistance of all the strains tested. Among matrix type, the one with only alginate (AM) gave the best performance while the core-shell system showed to be suitable for GI delivery of living cells resulting in the highest level of protection. The chitosan-alginate complex showed to be suitable for GI delivery of probiotic strains. Our findings suggested that microencapsulation might be a powerful tool to confer the important prerequisite that is the gastrointestinal resistance to strains with promising technological and functional properties.

Notes:

Due to the encouraging results exposed in this Chapter, experiments about the use of alginate core-shell microcapsules for probiotic delivery in the GI tract are going to be scaled up in collaboration with the Food Quality and Design group of Wageningen University, Netherlands. The behavior of *Lb. reuteri* provided in encapsulated form will be study during two weeks of trial in a Simulator Human Intestinal Microbial Ecosystem (SHIME[®]) consisting of five reactors for simulation of the stomach (acid conditions and pepsin digestion), the small intestine (digestive processes) and the ascending, transverse and descending colon (microbial processes), previously adapted with human faeces to simulate the human intestinal microbiota. According to a tentative plan, the main aims of the experiment will be the evaluation of i) the destiny of the microcapsules in intestinal compartment (e.g. site of their disruption and cell leakage) through microscopical observation and fluorescence *in situ* hybridization (FISH) of encapsulated *Lb. reuteri* for the comprehension of cell leaking kinetics, ii) the influence of probiotic *Lb. reuteri* on intestinal microbial dynamics through high throughput sequencing and iii) selective quantification of *Lb. reuteri* by qRT-PCR.

CHAPTER 6

Food functionalization by microencapsulated probiotics: three case studies

6.1 Introduction

The use of microencapsulation as strategy for the improvement of probiotic robustness in harsh conditions accounts many successful *in vitro* applications aimed to capacitate encapsulated probiotics to cope with stress induced by high osmotic pressure, high temperature conditions, low pH, complex microflora and gastrointestinal environment. Alongside with the aforementioned positive effects, one prerogative of microencapsulation technique, and probably the most important one, is to contribute to the development of new probiotic food carriers as well as to ameliorate the quality of marketed foods that are considered conventional vehicles of probiotics (i.e. yoghurt and fermented milks). Indeed, as already discussed, a global exploration of probiotic products on the market revealed that probiotic strains exhibit little or no survival in final goods, showing cell loads lower than that they are labeled (Corona-Hernandez et al., 2013; Raeisi et al., 2013; Chen et al., 2014). Most of the probiotic food products are categorized as functional foods and comprise between 60 and 70% of the whole functional food market (Tripathi and Giri, 2014). A recent global industrial analysis predicted that the acceptance of probiotic products among the population is continuously increasing to account about 34 billions € in 2018 and that milk and milk derivatives sectors, mainly with yoghurt and fermented milks, are undoubtedly leading this share of market (Global Industrial Analysis, 2013). In the optic of the growing consumption of probiotics, the development of non-dairy probiotic foods such as fruit juices that are lactose-free, soy-free and vegan-compliant, is becoming of paramount importance for the health-centric consumers that can suffer from lactose intolerance, are following a specific diet or that simply dislike milk and its derivatives (Gawkowski and Chikindas, 2013). The application of microencapsulation is driving the research development of new categories of probiotic food products. At this purpose, I recently reviewed scientific works dealing with the application of microencapsulated probiotics in different food matrices discovering that the most investigated categories are, in growing order, the ones of milk derivatives, fruit and vegetable based products, meat based products and finally the one of bakery products (De Prisco and Mauriello, 2016). This Chapter reports the inclusion of encapsulated probiotics in three different food matrices for their functionalization. These different food systems such as cheese, almond milk syrup and blueberry juice, have also been included in this research to test in real food systems the efficiency of some of the microcapsules we designed to protect probiotics under specific harsh conditions, namely thermal stress (Mozzarella cheese), osmotic stress (almond milk syrup) and stress from combination of low pH and presence of organic acids (blueberry juice).

6.3 First case study. Probiotication of Water- Buffalo Mozzarella cheese with microencapsulated *Lactobacillus plantarum* LMG P-21021

Milk derivatives as yoghurt and fermented milks have been widely used as delivery systems for probiotic cultures. Yet despite also cheese represents a suitable vehicle for delivering of probiotics in human intestine, both for its matrix that offer a high protection to living cells and for its compliance with worldwide long-term diet (De Prisco and Mauriello, 2016). If on one side cheese offer a suitable environment for the long term probiotic survival, for example because of their higher pH (4.8-5.6) than that of fermented milks (pH 3.7-4.5) or their reduced permeability to oxygen, on the other side their production poses some challenges to probiotic viability for the effect of technological factors, presence of natural antimicrobials, natural microbiota of some cheeses. As also recently reviewed by Feuch and Kwak (2013), the use of microencapsulation contributes to the development of many different types of cheese including Cheddar and low-fat Cheddar, White Iranian brined cheese, Kasar, Feta cheese and others (Feuch and Kwak 2013; De Prisco and Mauriello, 2016). Few studies instead have considered the inclusion of probiotics in “pasta filata” cheese, where the most relevant hurdle is certainly represented by the high temperature reached during curd stretching (Ortakci et al., 2012; Minervini et al., 2012; Rodríguez-Huezo et al., 2014). This cheese typology, obtained by cow (Fior di Latte cheese) or water-buffalo (Mozzarella cheese) milk is manufactured through chemical acidification of the curd or biological acidification obtained by using natural whey starter cultures or commercial starter cultures. Then, the acidified curd is undergone to stretching procedure through the use hot water and finally temperature of stretched and molded curd is cooled down by its immersion in cold water. Fior di Latte and Water- Buffalo Mozzarella cheese have a long traditional use in Italy and especially in the South of Italy, where they are fully integrated in the diet (Mauriello et al., 2002). Obviously, in the production of a probiotic “pasta filata” cheese, the addition of probiotics prior curd stretching cannot be by-passed and, in this optic, microencapsulation can be a powerful tool for sustaining probiotic viability against high temperature applied. Here following the manufacturing of probiotic Water- Buffalo Mozzarella Cheese in collaboration with a local cheese factory (La Tramontina, Cava dei Tirreni - Salerno) obtained with the use of microencapsulated probiotic lactobacilli is reported.

6.2.1 Methodological plan

6.2.1.1 Selection of heat resistant probiotic strain

Six probiotic strains such as *Lactobacillus plantarum* LMG P-2102, *Lactobacillus paracasei* LMG P-21021, *Lactobacillus rhamnosus* DSM 16605, *Lactobacillus acidophilus* LMG P-21381 from Probiotal collection, *Lactobacillus reuteri* DSM 17938 (previously isolated from Reuterin®) and *Lactobacillus plantarum* ATCC: SD 5209 (previously isolated from Equilibria®) were included in this study. They were preliminary tested for the ability to resist under thermal stress condition. At this purpose, all the strains were grown in MRS broth (Oxoid) at 37°C in aerobic conditions and counted on MRS Agar at the same condition of incubation. To select the most heat-resistant strain and submit it to microencapsulation process for further inclusion in

Mozzarella cheese, all probiotic strains were exposed to heat treatment of 65°C for 5 min. Overnight liquid cultures of each probiotic strain were prepared, then they were centrifuged at 5000 g for 10 min for the obtainment of cell pellets. After their washing with Ringer, cell pellets were resuspended in Ringer to restore the cell concentration of initial cultures to be further inoculated in ratio 1:10 into preheated (65°C) UHT skimmed milk. Thermal treatments were carried out at 65°C x 5 min in a hot water bath (SWB-20, Major Science). At the end of each treatment, samples were decimally serially diluted in Ringer solution and subsequently counted on MRS agar for the determination of cell viability calculated as log CFU/ml reduction by subtracting cell load after treatment from the cell load before heat treatment. Results showed that *Lactobacillus plantarum* LMG P-2102 was the most heat resistant and as such was used in subsequent experiments.

6.2.1.2 Microencapsulation of probiotic strain

Lactobacillus plantarum LMG P-2102 probiotic strain selected on the basis of heat resistance under subsection 6.2.1.2 above was encapsulated in chitosan coated alginate core-shell microcapsules (CAM) since, from preliminary *in vitro* investigation reported in Chapter 3, this microcapsule system was selected as the best in protecting living cells under thermal stress. Encapsulation of *Lactobacillus plantarum* LMG P-2102 was performed by vibrating technology according to what reported in details in Chapter 3 for preparation of cell cultures, encapsulating matrices, capsule preparation, coating procedure and determination of microcapsule cell load.

6.2.1.3 Mozzarella cheese manufacturing with free and encapsulated *Lb. plantarum* LMG P-2102 strain

For these trials, encapsulated *Lb. plantarum* was directly added to towed curds, previously obtained by fresh buffalo milk, and provided by La Tramontina. A total amount of 30 Kg of curds was divided into three portions and cut into small pieces (around 5 cm³ each). Then free cell suspension or microcapsules of *Lb. plantarum* (both with a total cell load of about 10¹² CFU) were added to two portions of the curd to assure a cell load of about 10⁸ CFU/g in the curd prior to the stretching procedure. For an optimal homogenization of free and encapsulated cells, the inoculated curds were continuously and manually stirred and grounded for 15 min. Aliquots (5 g) of each curd samples (C1: no probiotic inoculation; C2: inoculated with free cells of *Lb. plantarum* and C3: inoculated with encapsulated *Lb. plantarum* cells) were collected for bacterial enumeration (prior to stretching). C1, C2 and C3 were separately stretched in an excess amount of hot water at 90°C for about 7 min and then they were molded in an automatic machinery to obtain standardized Mozzarella cheese with a diameter of 8 cm. The temperature of the curds at the end of stretching procedure ranged always between 60-61°C. Finally, Mozzarella cheese samples were placed in a stainless steel mold and immersed in cold brine (10°C) containing 50 g/kg of NaCl for 2 h.

Mozzarella cheese aliquots were referred as M1, M2 and M3 for the samples obtained from C1, C2 and C3, respectively. The temperature of M1, M2 and M3 measured in 10 samples decreased to about 35°C in 30 min, 24°C after 1 h, and to 14 °C by the 2 h. After that, five aliquots of 5 g/each of M1, M2 and M3 were sampled for bacterial enumerations. They were performed by decimal serial dilutions of M1 and M2 in Ringer while M3 was decimally serially diluted in 0.2 M sodium citrate to favor the cell release from the capsules. The presence of

probiotic *Lb. plantarum* was evaluated by comparing the results of viable counts of not-inoculated samples (used as control) with the viable counts of samples inoculated with free and encapsulated *Lb. plantarum* and by microscopic observation of typical colonies.

6.2.1.4 Statistical analysis

Two independent productions of M1, M2 and M3 were carried out and five aliquots of each sample (C1, C2, C3, M1, M2 and M3) were used for viable counts. Results are expressed as averaged values of experimental replicas and a t-test analysis (Microsoft Excel for Mac version 11.5) was performed to ascertain significant differences between averages. Significance was declared at $P < 0.05$.

6.2.2 Results and discussion

6.2.2.1 Thermal resistance of probiotic strains

A preliminary screening based on thermo-resistance properties of six different probiotics was performed in order to select the most resistant strain to be used in Mozzarella cheese making. This selection was carried out in order to obtain the best performing couple probiotic/microcapsules system for further experiments. After the treatment at 65°C for 5 min *Lb. plantarum* LMG P-2102 population showed the highest level of survival, accounting a reduction of 3.40 ± 0.15 log CFU/ml, with respect to other probiotic strains tested (data not shown).

6.2.2.2 Survival of free and encapsulated *Lactobacillus plantarum* during Mozzarella cheese manufacturing

Free and encapsulated probiotic cells of *Lb. plantarum* were used for functionalization of Mozzarella cheese. The addition of probiotics to “pasta filata” cheese accounts few applications (Ortakci et al., 2012; Minervini et al., 2012; Rodríguez-Huezo et al., 2014). Ortakci et al. (2012) and Rodríguez-Huezo et al. (2014) made use of encapsulation to efficaciously produce “pasta filata” cheese such as Water- Buffalo Mozzarella and Mexican Oaxaca cheese, respectively. Differently, Minervini et al. (2012) selected some probiotic strains that gave a positive response to previous treatment of thermic pre-adaptation to produce probiotic Fior di Latte Mozzarella cheese. From our previous investigations we selected the chitosan coated alginate core-shell system as the best one for thermal protection of probiotic *Lb. reuteri* cells. Furthermore, we considered the dimension of core-shell microcapsule system, attested around 300 μm , suitable for the inclusion into Mozzarella cheese. Higher viable counts recorded for Mozzarella cheese samples with microencapsulated probiotic cells (M3) with respect to plain (not inoculated) Mozzarella (M1) and Mozzarella with free probiotic (M2) suggested higher survival level of microencapsulated strain. Results are reported in Tab. 6.2.1.

Tab. 6.2.1 Viable counts of not inoculated curd and Mozzarella, curds with free and encapsulated probiotic cells and resulting probiotic Mozzarella cheese with free and encapsulated *L. plantarum*

Samples	Curds	Mozzarella cheese
	Log UFC/g	
Not inoculated	6.80 ±0.2 ^a	5.10 ±0.3 ^a
Free <i>Lb. plantarum</i>	8.10 ±0.22 ^b	5.90 ±0.3 ^b
Encapsulated <i>Lb. plantarum</i>	8.15 ±0.20 ^b	7.20 ±0.2 ^c

Differences between mean values were attested by *t-test* and different letters among samples in the same column indicate significant differences. Significance was declared at $P < 0.05$.

Furthermore Fig. 6.2.1 showed that microcapsules were homogeneously “entrapped” in the curds.

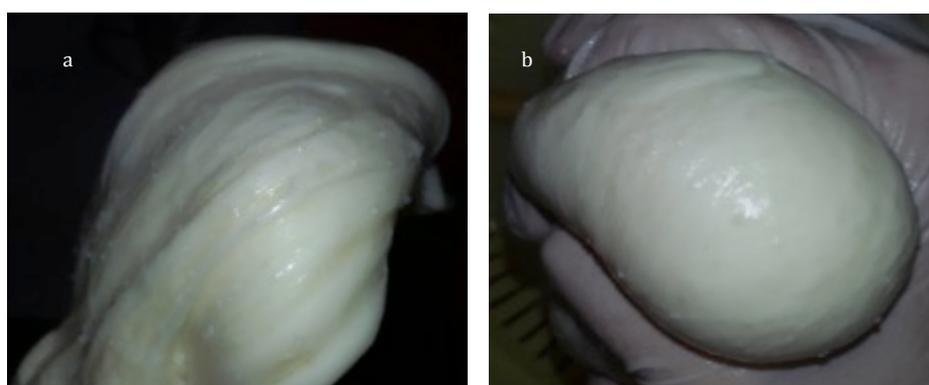


Fig. 6.2.1 Stretched curd and Mozzarella cheese inoculated with core-shell microcapsules of *Lactobacillus plantarum* LMG P-2102

6.3 Second case study. Probiotication of almond milk syrup with microencapsulated *Lactobacillus plantarum* LMG P-21021 and *Lactobacillus reuteri* DSM 17938

Almond milk syrup is a product that comes directly from the almond milk, added with sucrose to obtain syrup that can be stored longer at room temperature. We assisted, within a private project, a small company of Neapolitan area producer of almond milk syrup for the preparation of the HACCP plan for the hazards analysis during the production and distribution of this product and to define the relative control measures. Almond milk syrup is characterized by a high percentage of sucrose (60%). For this reason we thought that this product could serve as real food system to test the viability of encapsulated probiotic under osmotic pressure. Thus, almond milk syrup was functionalized by the addition of two strains of probiotic lactic acid bacteria, *Lactobacillus reuteri* DSM 17938 and *Lactobacillus plantarum* LMG P-21021, that were encapsulated in chitosan coated alginate matrix (AM) and core-shell (CAM) microcapsules and the capability of microcapsules to enhance probiotic resistance

under osmotic stress conditions was evaluated. As side effect we contributed to the possible development of a hedonistic probiotic food such as the almond milk based-beverage.

6.3.1 Methodological plan

6.3.1.1 Encapsulation of probiotic strains

Microencapsulation of *Lactobacillus reuteri* DSM 17938 and *Lactobacillus plantarum* LMG P-21021 and further determination of microcapsule cell load was performed according to the procedure described in Chapter 4 for the production of AM and CAM.

6.3.1.2 Evaluation of probiotic viability in almond milk syrup

To test the probiotic strain survival under osmotic stress condition, *Lactobacillus reuteri* and *Lactobacillus plantarum* cells in free (pellets from fresh cultures) and microencapsulated form were inoculated in the ratio 1/10 in almond milk syrup. Samples were stored at 20°C to simulate the real product storage conditions. All samples were analyzed for the viable counts immediately after the inoculum (T0) and weekly for a period of 1 month. Samples of almond milk syrup with added probiotic microorganisms in free and microencapsulated form were also monitored for pH values.

6.3.1.3 Statistical analysis

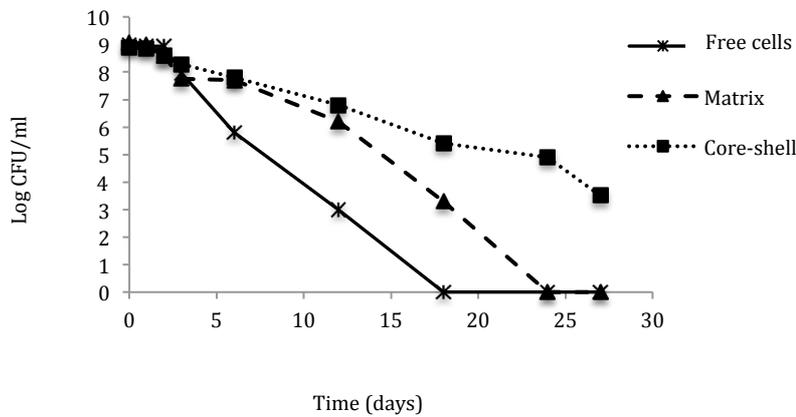
Three independent experiments were carried out and viable counts were determinate in duplicate. Results are expressed as average values \pm standard deviations.

6.3.2 Results and Discussion

6.3.2.1 Free and microencapsulated probiotic cell viability and in almond milk syrup

Lactobacillus reuteri DSM 17938 and *Lactobacillus plantarum* LMG P-21021 cells were inoculated in almond milk syrup in free and microencapsulated form in order to evaluate the resistance of the strain under conditions of osmotic stress and the protection conferred by two different microcapsules systems. As reported in Fig. 6.3.1, free probiotic *Lb. plantarum* survived better in the almond milk syrup than *Lb. reuteri* since no survival of *Lb. reuteri* cells was observed 15 days of inoculum in almond milk syrup. Differently, *Lb. plantarum* was still alive in the product with an average load of 4.3 log cycle after 35 days of inoculum. The resistance of both strains was enhanced by the microencapsulation (Fig. 6.3.1), however core-shell microcapsules conferred a higher level of protection than matrix microcapsules. Indeed, while *Lb. reuteri* cells in matrix microcapsules were no more detected after the timepoint at 20 days from the inoculum, they were still alive in almond milk syrup at the end of observation period.

a



b

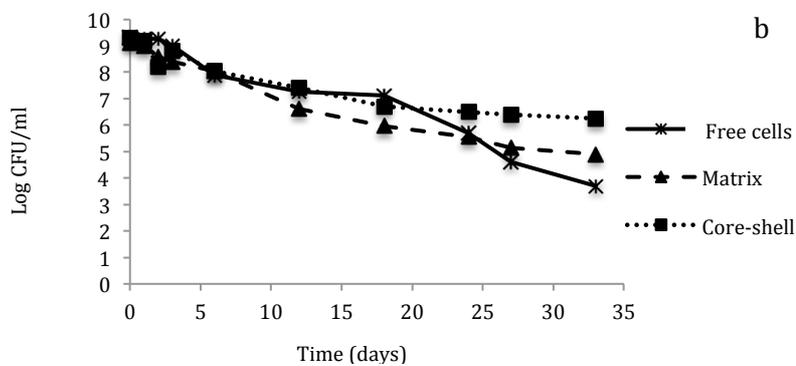


Fig. 6.3.1 Viable counts of free and encapsulated *Lb. reuteri* (a) and *Lb. plantarum*(b) inoculated in almond milk syrup. Results are expressed as averaged values of three independent experiments carried out in duplicate. Standard deviations were always <0.15

A slow penetration of sucrose inside core-shell microcapsules due to the external alginate layer might explain the higher survival of probiotics when protected by this microcapsule system. Results from D’Orazio et al., 2015 confirmed our findings. Indeed they demonstrated that chitosan coated alginate microcapsules efficiently protect probiotic *Lb. plantarum*PBS067 cells from osmotic pressure given by a solution of 30% isoglucose or glucose-fructose syrup. The inoculum of both *Lb. plantarum* and *Lb. reuteri* in free and encapsulated probiotic lactobacilli did not lead to a reduction of the pH that remained stable at a value of 5.7 ± 0.1 throughout the analysis period. In general, to the extent of our knowledge, very few papers addressed the exposure of microencapsulated probiotics to osmotic stress conditions.

6.4 Third case study. Probiotication of blueberry juice with microencapsulated *Lactobacillus reuteri* DSM 17938

Development of fruit and vegetable-based probiotic foods is closely related to the increasing demand of products with low cholesterol content and free from animal derivatives and milk allergens (Céspedes et al., 2013). In this optic, fruit- and vegetable-based beverages are an attractive choice because of i) consumer preference towards convenience, healthy diet and natural ingredients; ii) naturally-occurring health-promoting components including vitamins and antioxidants; iii) possibility to meet consumer demands for container contents, size, shape, and appearance; iv) opportunity to incorporate desirable nutrients and bioactive compounds;

v) their taste profiles that are pleasing to all the age groups; ii) they are perceived as being healthy and refreshing (Prado et al. 2008; Sun-Waterhouse 2011; Wootton-Beard and Ryan 2011). Despite the above-mentioned considerations and recent food trends related to vegetarianism and to soy allergy and lactose intolerance, the availability of 100% probiotic fruit- and vegetable-based beverages is still limited (Gawkowski and Chikindas, 2013) and this encourages researchers to investigate this food category for functionalization with probiotics (Ferreira et al. 2005; Pereira et al., 2011; Costa et al., 2013). On the other hand, low pH of fruit juices, typically ranging between 2.5 and 3.7, combined with the effect of phenolic acids (benzoic acid) and lactones, available in high amounts in some fruit juices (e.g. cranberry juice), could be responsible of a prominent loss of probiotics viability (Vinderola et al., 2002; Sheehan et al., 2007).

What is following described as the case of functionalization of blueberry juice by the addition of probiotic lactobacilli is a part of a wider research finalized at the development of new vegetable-based probiotic carriers. In the above mentioned study we evaluated i) the ability of six probiotic strains (selected among homo- and heterofermentative lactobacilli) to grow and to ferment six fruit-based matrices (i.e. pineapple, apple, orange, blueberry, grapefruit and exotic fruit juices); ii) their resistance during the storage of fermented fruit juices at 4 and 20°C to determine the recommended storage condition and iii) the sensorial analysis, in collaboration with Prof. Di Monaco (Dept. of Agricultural Sciences, University of Naples), by Napping and modified Flash Profile (Liu et al., 2016) of three fermented fruit juices previously selected for the sensory analysis. We did not include in the sensorial test all the fruit juices that did not show any sensorial modifications after the addition of the probiotic strains during a preliminary informal sensorial analysis and that exhibited poor probiotic growth and survival as mentioned earlier. Since *Lactobacillus reuteri* DSM 17938 showed the weakest ability to ferment and to survive in blueberry juice, it was encapsulated in two different microcapsule systems namely alginate matrix and chitosan-coated alginate matrix microcapsules in order to increase its resistance to low pH and blueberry juice components (e.g. organic acids), assessing the protective effect given by the alginate and chitosan toward probiotics.

6.4.1 Methodological plan

*6.4.1.1 Microencapsulation of *Lb. reuteri* DSM 17038*

Lactobacillus reuteri DSM 17938 was previously selected for this study because of its native weak ability to grow and to survive in blueberry juice. With the aim to enhance its resistance in the above mentioned food matrix, it was encapsulated in alginate and chitosan-alginate matrix microcapsules by vibrational nozzle technology. Cell culturing, preparation of these microcapsules, their counting and conservation was performed as widely described in Chapter 3.

*6.4.1.2 Inoculum of free and microencapsulated *Lb. reuteri* DSM 17038 cells in fruit juice*

Aseptic and ambient-stable biological blueberry juice (commercial label) having a pH of 2.9 ± 0.1 was used for its functionalization with probiotic microorganisms. At this purpose, *Lb. reuteri* cells were added in blueberry juice in free and encapsulated form. Prior to addition to fruit juice, free cells for the inoculum were obtained by the overnight cultures that were

centrifuged at 5500 g for 10 min., separated from MRS supernatant and finally resuspended using fruit juice to restore the cell concentration of the initial cell culture. Also microcapsules were let to spontaneously sediment and, after discarding of Ringer solution, were resuspend using fruit juice to restore the initial cell concentration of microcapsule samples. Free cells and cells in alginate and chitosan-coated alginate microcapsules were inoculated in fruit juice to reach a cell concentration of 7.05 ± 0.10 Log CFU/ml. Inoculated juices were incubated at 37°C for 20 hours to evaluate a possible cell growth when in encapsulated form and they were then stored at both 4°C for 20 days for shelf life evaluation. At specific stages during the experiments, microcapsules were observed with optical microscope in bright field (Y-FL Nikon) with a magnification of 40X to verify the integrity of the structures and to assess the possible presence of cells released from microcapsules.

6.4.1.3 Statistical analysis

Three independent productions of probiotic fruit juices were carried out and viable counts were determined in duplicate. Results are expressed as average values of experimental replicas and a t-test analysis (Microsoft Excel for Mac version 11.5) was performed to ascertain significant differences between averages. Significance was declared at $P < 0.05$.

6.4.2 Results and discussion

Results about the growth and viability of free and encapsulated *Lb. reuteri* in blueberry juices are reported in Fig. 6.4.1. As already evaluated, free *Lb. reuteri* did not grow in fruit juices and exhibited the weakest viability with the respect to the microencapsulated counterparts.

In line with several papers dealing with the use of microencapsulation technique to protect probiotic in fruit-based matrix (De Prisco and Mauriello 2016), results obtained by inoculating the probiotic strain in encapsulated form indicated a higher cell survival (Fig. 6.4.1). However different resistance profiles were observed between *Lb. reuteri* encapsulated in alginate and in chitosan alginate matrix. Accordingly, in first analysis a weak but significant ($P < 0.05$) increase in the cell load was detected suggesting a weak ability of the strain to growth in blueberry juice when encapsulated in alginate microcapsules. Alongside, no significant reduction of the initial pH was observed. This increasing of cell population could be attributed to a dual effect of alginate able to protect bacteria from low pH characteristic of the juice and in the same time to permit the efflux of nutrients inside the capsules. Differently, when the capsules were coated with chitosan, any increasing of *Lb. reuteri* population was recorded, indicating a higher barrier effect of coated alginate matrix. By contrast, just this higher barrier effect of chitosan-coated alginate microcapsules was likely responsible of the higher viability of *Lb. reuteri* cells along the storage in fruit juice (Fig. 6.4.1). The greater permeability of alginate microcapsules to fruit juice matrix is also suggested by the observation of the microcapsules after 7 days from the inoculum in blueberry juice. Indeed Fig. 6.4.2 shows that alginate capsules appeared more coloured than chitosan coated microcapsules for the effect of the blueberry matrix.

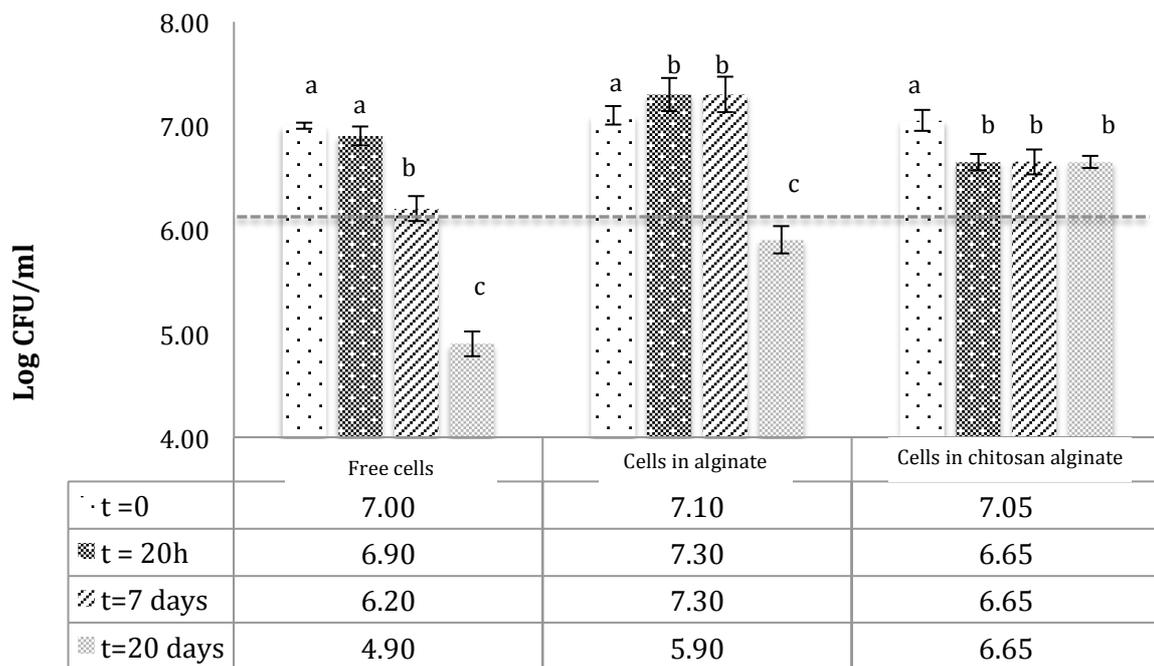


Fig. 6.4.1 Viable counts of *Lb. reuteri* cells in free form and encapsulated in alginate and chitosan coated alginate matrix microcapsules during growth (t=20h) in blueberry juice at 37°C and further storage for 20 days at 4°C. Results are expressed as average values from three experiments carried out in duplicate ± standard deviations. Differences between mean values were attested by t-test and different letters among the same sample indicate significant differences at a level of $P < 0.05$

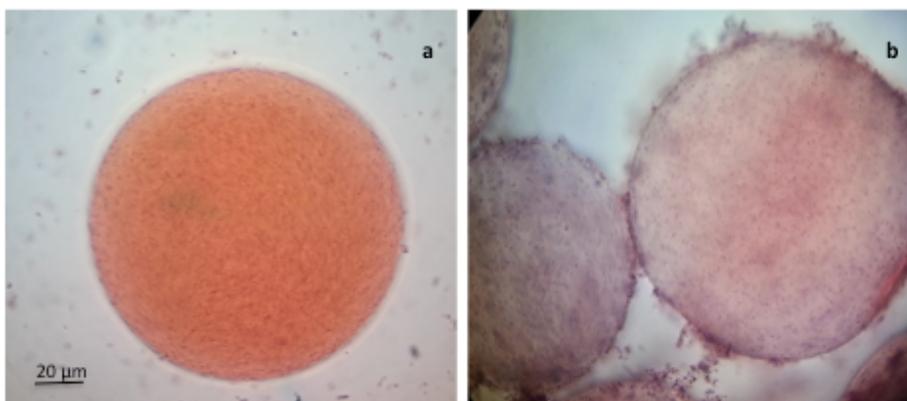


Fig. 6.4.2 Alginate (a) and chitosan coated alginate capsules of *Lb. reuteri* after 7 days of inoculum in blueberry juice

As suggested by Truelstrup-Hansen et al. (2002) microcapsules having a size below 100 μm could limit negative sensory impact in foods. Matrix morphology was selected for this experiments because of the small size (around 110 μm) of this microcapsule system with respect to core-shell morphology, to attempt to avoid the perception of microcapsules during the consumption of a liquid food product.

6.5 Conclusion

Probiotication of different food matrices, including fruit and vegetable-based beverages, baked and thermal treated products and other food categories as well as the use of probiotic bacteria as starter culture is aimed at broadening the availability of probiotic foods, in order to spread

the conscious and unconscious consumption of these beneficial microbes. The frame presented in this Chapter strongly encourages the research and the industrial development of foods enriched with encapsulated probiotics, since microencapsulation showed to be able to sustain probiotic viability against hurdles encountered during foods production and during their storage. In this optic, in order to better understand the behavior and potentiality of the different encapsulating systems, the study of solute diffusion kinetics into the capsules and the determination of the acceptance of food products functionalized with probiotic microcapsules should be better addressed.

CHAPTER 7

Behaviour of microencapsulated starter culture during yoghurt manufacturing

7.1 Introduction

Microencapsulation is a widely known technology to enhance bacterial viability and functionality. It is used for preservation of probiotic viability during food processing in order to ensure the generally recommended dose of at least 10^7 CFU/g or ml of food along the whole shelf life of the product (Corona-Hernandez et al. 2013). Microencapsulated probiotics have been reported to resist gastrointestinal (GI) conditions, allowing them to reach the lower gut and deliver their health-related functionalities (Cook et al. 2012). Microencapsulation may also improve cell fermentation performance. Either in its early form of immobilization and in the evolved microencapsulation, the use of these technologies has been widely explored in the past to protect cells from external contamination and to achieve an easier cell separation from fermentative media (Rathore *et al.* 2012). The use of immobilized/encapsulated cells in batch fermentation proved to enhance the production and recovery of metabolites (e.g. ethanol, lactic acid, riboflavin), and cells could also be effectively recycled (Westman *et al.* 2012). Similarly, when applied in the dairy sector the immobilization of lactic acid bacteria led to higher cell density, protection from oxygen and phage contamination and better cell survival during freeze-drying of starter cultures (Champagne et al. 1994; Mirzaei et al. 2012). However, some aspects of bacterial microencapsulation such as the metabolism of cells and their behaviour in leading food fermentation are still not fully investigated. When encapsulated, cells are exposed to new physico-chemical conditions created by the capsules themselves, mostly given by the closer cell-cell and cell-metabolites contact and by the lower diffusion rate of nutrients and gases (i.e. oxygen) into the capsules. The resulting microenvironment may lead to an alteration of cell metabolism, likely influencing some specific metabolic traits (Doleyres et al. 2004; Junter et al. 2002). For instance, a metabolic shift from homo- to heterolactic fermentation has been reported in immobilized lactobacilli during repeated batch cultivation (Krishnan et al. 2001; Thomas and Turner, 1981), an increased cell tolerance to stress factors (Doleyres et al. 2004; Krisch et al. 1997; Trauth et al. 2001) or an activation of the primary metabolic function of immobilized yeasts (Junter et al. 2002). Recently, the use of encapsulated microorganisms in food fermentation is regaining attention (Bilenler et al. 2017; Gallo et al. 2014) in the wake of evidences about the protective effect of immobilization procedure widely reported in the past (Champagne et al. 1994; Kearney et al. 1990) and that some strains have better fermentation performances when in encapsulated form (Nedović et al. 2015). The wine and brewing sectors have promptly caught the potential of encapsulated/immobilized yeasts. Indeed, Nedović et al. (2015) recently highlighted the

advantages of such application, including higher volumetric cell density as well as aroma-related metabolites and the increased cell ability to withstand the toxicity of fermentation environment. In this framework, it is logical to explore the encapsulation of well-know probiotic cultures to i) seek their use in fermentation as starter cultures and ii) to strength probiotic culture against food-related stress factors and further digestion (Corbo et al. 2013; Krunić et al. 2015). Because of the impact of new microenvironment on microbes the study of cell metabolites patterns such as those related to aroma formation might be a preliminary step toward the elucidation on the possible effect of microenvironment on the physiology of entrapped bacterial cells.

In this work a mix of *Streptococcus thermophilus* and a probiotic strain of *Lactobacillus delbrueckii* was encapsulated in two different systems, matrix and core-shell capsules, made by alginate and chitosan. Starter cultures in free (non-encapsulated) and in both microencapsulated forms were used for production of set-yoghurt with the purposes of investigating the effect of the capsules on cell growth and primary metabolic functions (acidification and proteolysis) and evaluating the protective effect of microcapsules on bacterial cells during yoghurt storage and simulated digestion. Finally, volatile organic compounds (VOCs) of milk fermented by free and encapsulated bacteria were compared.

7.2 Materials and Methods

7.2.1 Microorganisms and culture conditions

The probiotic *Lactobacillus delbrueckii* subsp. *bulgaricus* strain was isolated from the commercial food supplement YOVIS® (Sigma-Tau) on acidified (pH 5.6) de Man Rogosa Sharp (MRS, OXOID Ltd., Basingstoke, Hampshire, England) and identified by sequencing of 16S rDNA tract according to Blaiotta et al. (2004). The strain was stored at -20°C in MRS broth added with glycerol and routinely cultured in acidified MRS broth for 24 h at 42°C under microaerophilic conditions (Anoxomat™-Mart® Microbiology, Drachten the Netherlands). Overnight cultures (8.10 ± 0.10 Log CFU/ml) were pelleted by centrifugation at 6500 rpm for 10 min, washed twice in quarter strength Ringer solution (Oxoid, below indicated with Ringer) and finally resuspended in half the volume of Nilac skimmed milk (NIZO, Ede, the Netherlands). Suspensions of *Lactobacillus delbrueckii* subsp. *bulgaricus* cells in skimmed milk were aliquoted in sterile vials and maintained at -45°C. *Streptococcus thermophilus* strain C44, supplied by CSK Food Enrichment (Ede, The Netherlands), was available in frozen pellets for direct vat inoculation and routinely and cultured overnight in M17 at 37°C under aerobic conditions.

7.2.2 Microencapsulation of starter culture

Strains were grown as previously described and then washed twice in Ringer. Resulting pellets were diluted in Ringer to obtain cell suspensions having an OD₆₀₀ of 2.2 and 2.0 for *Lactobacillus delbrueckii* and *Streptococcus thermophilus*, respectively, corresponding to a cell density of 8.20 ± 0.20 Log CFU/ml for both strains, as preliminarily assessed. Suspensions were mixed and a single pellet was obtained by centrifugation, suspended in an equal volume of 1.6% alginate solution and used to produce matrix microcapsules and core-shell microcapsules by vibrational nozzle technology implemented on (Encapsulator B-395 Pro BÜCHI

Labortechnik, Flawil, Switzerland). Detailed explanation of this technology is reported in De Prisco et al. (2015). Matrix microcapsules were produced by a single extrusion (80 μm nozzle) of alginate-cell suspension fed at a rate of 3 ml/min with the syringe pump. Values of membrane vibration frequency and electrical field were 1900 Hz and 1200 V, respectively. For production of core-shell microcapsules, a simultaneous-double extrusion was carried out through a concentric nozzle system, measuring 80 and 200 μm for core and shell extrusion, respectively. Cells were entrapped only into the core obtained with 1.6% alginate solution. Instead, shell was produced with a 1.5% alginate solution fed through the pressure bottle system at 400 mbar. Values of membrane vibration frequency and electrical field were of 1900 Hz and 2500 V, respectively. In both processes, droplets were collected in a 0.5 M CaCl_2 solution bath for polymerization of alginate that causes microcapsule formation. After sedimentation and discarding of the supernatant, microcapsules were washed twice in Ringer and then coated with 0.8% chitosan solution as previously described (De Prisco et al. 2015). They were let to sediment, washed again in Ringer for removal of all chitosan solution and finally suspended in double of the volume (w/v) of Ringer to be stored at 4°C. Chitosan coated matrix and core-shell microcapsules are named AM and CAM, respectively. If not differently specified, all chemicals used were purchased from Sigma Aldrich.

7.2.3 Enumeration of microencapsulated cells

Samples containing microencapsulated cells were plate counted in original form (intact microcapsules) or after microcapsule disruption. They were decimally serially diluted in Ringer or 0.2 M sodium citrate to conserve intact or to promote disruption of microcapsules, respectively. Sodium citrate was used to degrade calcium alginate network and promote cell release (Gombotz and Wee, 2012). For counting of *Lactobacillus delbrueckii* cells, dilutions were pour-plated on MRS agar (pH 5.6) and incubated at 42°C for 48 h under microaerophilic conditions. *Streptococcus thermophilus* cells were counted by spread plate technique on M17 agar incubated aerobically at 37°C for 24 h.

7.2.4 Set yoghurt preparation by free and encapsulated starter culture

Nilac skimmed milk powder was reconstituted at 10% (w/v) in milli-Q water at 50°C, pasteurized in batches of 400 ml at 90°C for 6 min and rapidly cooled at 42°C. Milk batches were inoculated with free cells from unfrozen stocks (15-20 min at room temperature) and with both microcapsule types to reach an initial concentration of starter culture of 6.45 ± 0.08 Log CFU/ml with a 1:1 ratio of the two microorganisms.

Milk pH (InoLab pH720, WIW, Weilheim, Germany) and bacterial growth were hourly monitored and fermentation stopped by fast refrigeration at 4°C when pH value of 4.6 ± 0.1 or a deadline of 10 hours were reached. Samples were stored at 4°C for 28 days.

7.2.5 Evaluation of proteolysis

Proteolysis was each hour monitored during milk fermentation using *o*-phthaldialdehyde (OPA) method (Mauriello et al. 1998). Briefly, 5 ml of sample were added of 2.5 ml of 0.75 M trichloroacetic acid, gently mixed, incubated for 10 min at room temperature and then paper filtered (Whatman Grade #2). One ml of OPA solution (25 ml of 0.1 M sodium tetraborate,

2.5 ml of sodium-dodecyl sulphate 20%, 0.04 g of OPA dissolved in 1 ml of methanol, 100 μ l of β -mercaptoethanol), used within 2 h after its preparation, was added to 50 μ l of filtered-fraction, mixed directly in an UV-transparent cuvette and incubated for 2 min at room temperature prior to absorbance determination at 340 nm. The absorbance of released free amino groups was read against L-serine standard curve (0.0- 0.4 mM). Not-inoculated skimmed milk was used as blank.

7.2.6 Analysis of VOCs by headspace Solid Phase Micro Extraction (SPME) and gas chromatography/mass spectrometry (GC/MS)

Fermentation was performed in GC vials under the same conditions described above. GC vials (10 ml, 46x22.5 mm) were sealed with 20 mm silicon/PTFE septa and magnetic caps (Grace, Albany, OR, USA) to avoid loss of volatile fraction components. At the beginning and at the end of the fermentation period, vials were stored at -20°C and thawed immediately before GC/MS investigation. Analysis of volatile compounds was performed according to the procedure of Hettinga et al. (2008) for milk and milk derivatives and modified by Settachaimongkon et al. (2014) for yoghurt. Volatiles were extracted from the headspace of vials by their absorption on a 75 μ m Carboxen™ PDMS-SPME fiber (Supelco, Bellefonte, PA, USA) operated by auto-sampling (TriPlus™-Autosampler, Thermo Scientific, Austin, TX, USA) for 5 min. at 60°C. Volatiles were thermally desorbed for 15 min from the SPME fiber in the GC injection port and then analyzed by GC/MS operated with Trace GC Ultra coupled to DSQ II mass spectrometer (Thermo Scientific, Austin, TX, USA). Volatile separation was performed with a Stabilwax® -DA-Crossband® -Carbowax® -polyethylene-glycol column (30 m length, 0.32 mm internal diameter, 1 μ m film thickness from Restek, Bellefonte, PA, USA) and helium, constantly fed at 1.5 mL/min, was used as mobile phase. Initial temperature of the oven was held at 40°C for 3 min, then it increased with a ramp of 15°C/min up to a final temperature of 220°C for 1 min. Mass spectrometer operated in the electron impact mode at 70 eV, ion source at 225°C and ionization had a voltage within a mass scan range of 33-250 m/z. For peak identification, volatile's spectra were analyzed by ADMIS software with the NIST/EPA/NIH database and a specific library for milk and milk derivatives provided by Hettinga et al. (2009). Peak integration with specific retention time and m/z was carried out with XCalibur software (Thermo Scientific, TX, USA). Concentration was expressed as log₁₀ of absolute peak area of each compound.

7.2.7 Evaluation of cell viability during yoghurt storage and simulated gastrointestinal (GI) transit

The bacterial viability during 28 days of yoghurt storage was weekly evaluated by enumeration of free and microencapsulated *Lactobacillus delbrueckii* and *Streptococcus thermophilus* cells. At the end of this period, yoghurt samples were digested to test the ability of the strains to survive. Counting of bacteria at the final time-point of yoghurt storage (t=28 days) was considered the initial bacterial population level in the test of survival under GI condition. The protocol for simulated gastrointestinal digestion was adapted from Vizoso Pinto et al. (2006). Briefly, five grams of each sample were incubated in ratio 1:1 with saliva simulating solution (SSS; 5 g/l NaCl, 2.2 g/l KCl, 0.22 g/l CaCl₂ and 1.2 g/l NaHCO₃, 100 mg/l lysozyme, pH 6.9) at 37°C for 5 min. Then, gastric simulating solution (GSS; 5 g/l NaCl, 2.2 g/l KCl, 0.22 g/l CaCl₂ and 1.2 g/l NaHCO₃, 3g/l pepsin, pH 2.5) was added to samples in1:4 ratio (referred to sample

volume) followed by incubation at 37°C for 120 min and manually stirring during 20 min. In the third phase, intestinal simulating solution (ISS; 6.4 g/l NaHCO₃, 0.239 g/l KCl, 1.28 g/l NaCl, 0.5% bile salts and 0.1% pancreatin, pH 7.0) was added to samples in ratio 1:5 to obtain a final 1:10 dilution of yoghurt samples that were incubated at 37°C for 1.5 and 3.0 hours. At these two final time points the viable counts of *L. delbrueckii* and *S. thermophilus* were determined in Log CFU/ ml of sample.

7.2.8 *Morphology of microcapsules*

Microcapsule morphology was determined by microscopy and changes were monitored during milk fermentation and simulated gastrointestinal digestion. Observations were made by using an optical microscopy (Axioskop Zeiss, Göttingen Germany) coupled to a digital camera (Axiocam HCR, Göttingen, Germany).

7.2.9 *Statistical analysis*

Three independent set-yoghurt productions were made in duplicate. A t-test analysis (Microsoft Excel for Mac version 11.5) was performed to ascertain significant differences between averages; significance was declared at P<0.05.

7.3 Results

7.3.1 *Microcapsules morphology and cell load*

The two types of microcapsules (AM and CAM) differed in structure and diameter. Optical microscope images of microcapsules after chitosan coating are presented in Figure 7.1. AM are depicted in Figure 7.1a: they had a diameter of $110 \pm 10 \mu\text{m}$ and the cells entrapped in this system were dispersed throughout the entire capsule and cells at the surface were directly exposed to the external environment. Instead, CAM microcapsules had a diameter of about $300 \mu\text{m}$ and cell loaded core was homogeneously covered by the alginate outer layer (Fig. 7.1b), which protects cells from the environment. Bacterial load was calculated before and after disruption of microcapsules and results are reported in Table 7.1. Microcapsules after fermentation are depicted in Figures 7.1c and 1d, showing that both type of capsules retain their morphology during milk fermentation.

7.3.2 *Cell growth and acidification kinetics during milk fermentation*

Fermentative ability and growth kinetics of free starter culture in milk was compared with that of starter culture in microencapsulated form. Results are shown in Figure 7.2. Free starter culture was able to ferment milk until pH 4.6 ± 0.1 in 6 hours and the number of cells increased about 2.1 log cycles within this time (Fig. 7. 2a). Cells encapsulated in AM required longer time to acidify milk prolonging the fermentation up to 10 hours (Fig. 7. 2b). Within this time cell population increased 2.3 log cycles reaching the same bacterial load of yoghurt prepared with free cells (Fig. 7. 2b). Encapsulation of starter culture in CAM delayed milk acidification more markedly. As shown in Figure 7.2c, after 10 hours pH had still a value of 5.2, which is not enough to obtain protein coagulation. However, an overall increase of 2.1 log cycles was registered for the cell population in these capsules.

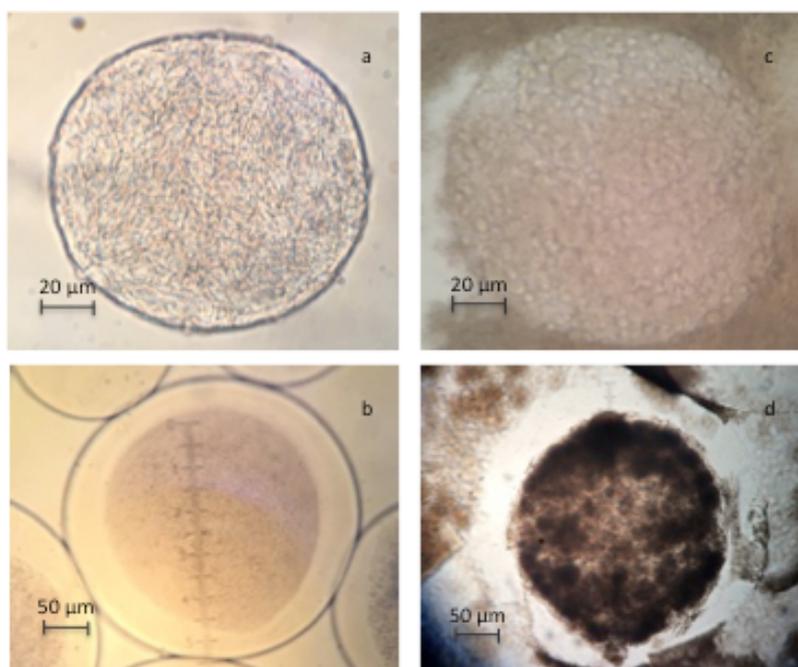


Fig. 7.1 Matrix (AM, panel a and c) and core-shell (CAM, panel b and d) capsules after the inoculum in milk (a and b, 40X magnification) and after 6 hours of fermentation (c and d, 10X magnification)

Table 7.1 Bacterial load (Log CFU/ml) of intact and disrupted matrix (AM) and core shell (CAM) microcapsules

	AM		CAM	
	<i>L. delbrueckii</i>	<i>S. thermophilus</i>	<i>L. delbrueckii</i>	<i>S. thermophilus</i>
intact	7.20±0.10	7.15±0.15	5.50±0.10	5.40±0.10
disrupted	9.05±0.05	9.00±0.05	8.10±0.20	8.20±0.15

Mean results of three independent trials ± standard deviation

7.3.3 Cell leaking from microcapsules during milk fermentation

Cell load of samples during milk fermentation, measured on intact and disrupted AM and CAM, are reported in Figure 7.3. Results show that the number of CFU/ml remains constant for 2 and 6 h for intact AM and CAM, respectively, and that a dramatic increase of the value was registered during the last 2-3 h of fermentation. On the other side, results of disrupted microcapsules show a similar growth kinetic in both AM and CAM (Fig. 7. 3). The gap between values of disrupted and intact microcapsules at each time indicates the magnitude of population remaining entrapped into the capsules during the milk fermentation.

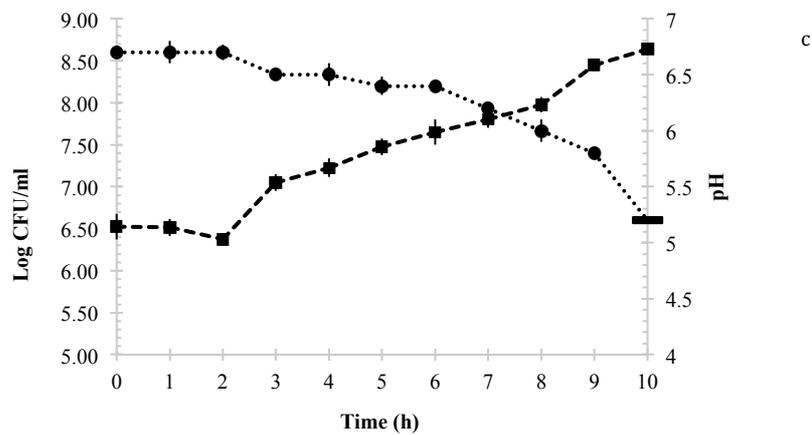
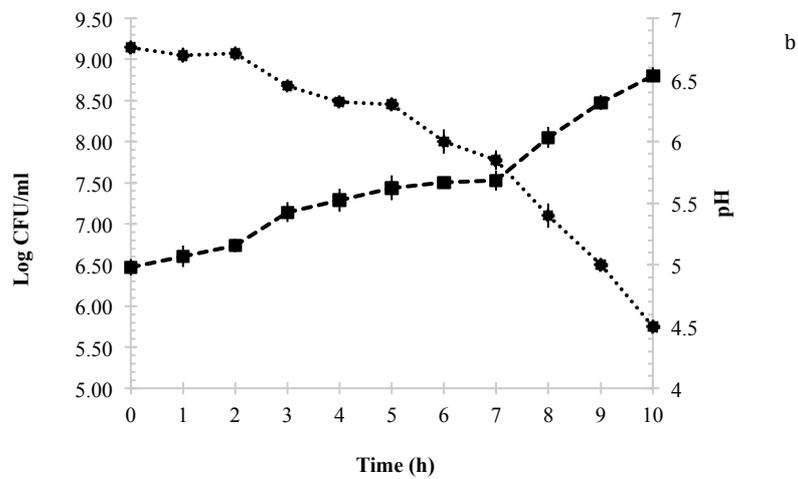
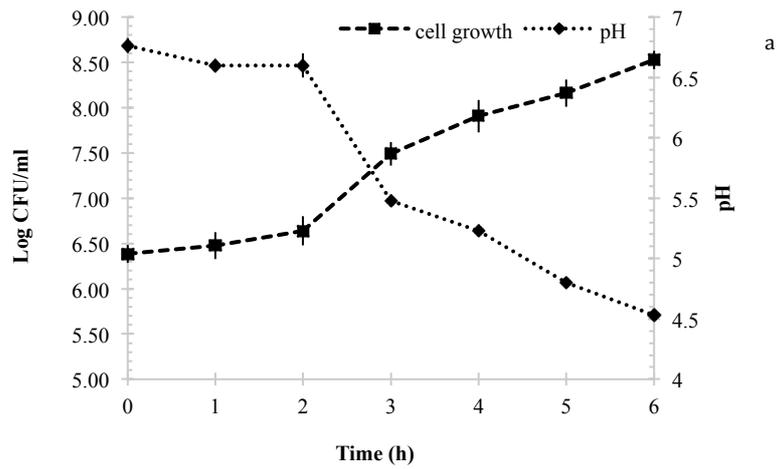


Fig. 7.2 Growth and pH kinetics during milk fermentation by yoghurt starter cultures in free form (a) and entrapped in matrix, AM (b) and core-shell, CAM (c) microcapsules. Bacterial loads are the sum of values separately acquired for *Lactobacillus delbrueckii* and *Streptococcus thermophilus*. Results are mean values of three different replicas of experiments carried out in duplicate, bars represent standard deviations

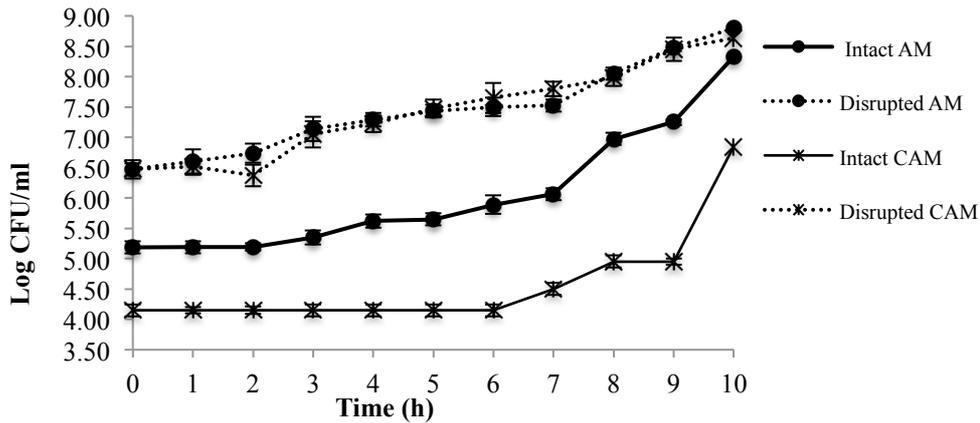


Fig. 7.3 Bacterial loads enumerated on intact and disrupted matrix (AM) and core-shell microcapsules (CAM) during milk fermentation

7.3.4 Evaluation of proteolysis

As shown in Figure 7.4 proteolysis was recorded in all samples during fermentation but with different kinetics between free and encapsulated bacteria. Higher concentrations of free amino groups were detected in samples fermented by free starter culture at each time points. After six hours of fermentation the optimal pH value (4.6 ± 0.1) was obtained and the concentration of free amino groups was about 0.23 mM for free *L. delbrueckii* and *S. thermophilus*. As observed for acidification profiles also the proteolytic activity was delayed when the bacteria are encapsulated both MM and CAM. Similar concentrations (0.24 mM) of free amino groups to the ones observed for free cells were reached by *L. delbrueckii* and *S. thermophilus* in MM only after 10 hours of fermentation. At the same time-point, in the yoghurt fermented by starter culture in CAM a lower degree of released free amino groups of 0.20 mM was recorded.

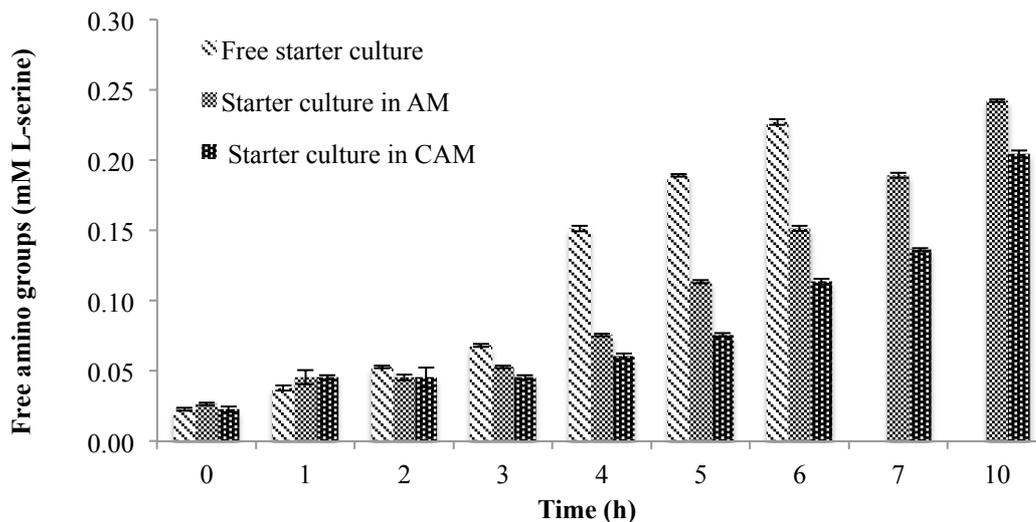


Fig. 7.4 Proteolytic activity of free and encapsulated *S. thermophilus* and *L. delbrueckii* in matrix (AM) and core-shell (CAM) microcapsules during skimmed milk fermentation at 42°C. Values are averaged from three independent trials carried out in duplicate \pm standard deviations

7.3.5 Volatile metabolites patterning

The comparative analysis of volatiles was done between the yoghurt obtained by conventional free culture and the one obtained with MM microcapsules as the acidification was not sufficient when milk was fermented by CAM. Results of table 7. 2 showed a pool of 28 volatile compounds (i.e. carbonyl and sulphur compounds, alcohols and organic acids) both in milk just after the inoculum and in yoghurt samples, with exception of 2,3 pentanedione and pentanoic acid, which were not detected in unfermented milk. Data analysis showed that: *i*) 6 compounds were found in unfermented milk and their concentration remained unaffected during milk fermentation; *ii*) 10 compounds significantly increased ($P < 0.05$) but equally in free and encapsulated cells ($P > 0.05$); *iii*) for 9 compounds the concentration was different in all samples ($P < 0.05$); *iv*) 2 compounds remained unaffected during milk fermentation with free cells ($P > 0.05$) but significantly increased in yoghurt containing encapsulated cells ($P < 0.05$); *v*) 1 compound remained unaffected during milk fermentation with encapsulated cells ($P > 0.05$) but significantly increased in yoghurt containing free cells ($P < 0.05$). The 13 compounds, whose concentration is affected by microencapsulation of cells, are highlighted in table 7. 2.

7.3.6 Starter cultures survivability during yoghurt storage and simulated digestion

Viability of starter cultures in free and microencapsulated form was monitored during 28 days-long storage of yoghurt at refrigerated condition. Free population of *S. thermophilus* and *L. delbrueckii* decreased from 8.40 and 8.65 Log CFU/ml to 3.00 and 6.70 Log CFU/ml, respectively, at the end of storage period. By contrast, microencapsulated starter culture showed a higher survivability since *S. thermophilus* and *L. delbrueckii* population were detected at 6.00 and 7.70 Log CFU/ml, respectively. Yoghurt samples were exposed to simulated digestion in order to evaluate viability after the exposure to salivary (SSS), gastric (GSS) and intestinal environments (ISS). Results reported in Figure 7.5 show that microencapsulated bacteria survived better than free cells to the digestive condition. At the first time-point (i.e. after 10 min in SSS, 2 h in GSS and 1.5 h in ISS), no survivors were found in both free *S. thermophilus* and *L. delbrueckii* population while the microencapsulated counterparts showed about 100% of survival. After additional 1.5 h in ISS, *S. thermophilus* and *L. delbrueckii* population survived at about 60% and 1.8%, respectively.

Along with bacterial survival during exposure to GI conditions we also monitored the performance of microcapsules in releasing cells. After exposure to salivary and gastric conditions, microcapsules did not lose their structure and still retain their original morphology. Differently, as shown in Figure 7.6 in intestinal simulating environment chitosan-alginate gel underwent a gradual swelling permitting a gradual release of cells.

Table 7.2 Concentration, expressed in \log_{10} of absolute peak area, of each compound detected by GC/MS analysis of unfermented milk and yoghurt obtained with free and encapsulated starter culture in matrix (AM) microcapsules.

Compound	RT (min)	Unfermented milk	Free cells	Encapsulated cells
acetaldehyde	2.20	5.45 ^A	7.74 ^B	7.62 ^B
dimethyl sulfide	2.50	6.11 ^A	6.15 ^A	6.32 ^A
acetone	3.23	7.40 ^A	7.51 ^A	7.84 ^B
1-heptanol	3.90	5.42 ^A	5.37 ^A	5.14 ^A
2-butanone	4.41	6.43 ^A	6.68 ^A	6.74 ^A
ethanol	4.97	4.54 ^A	6.16 ^B	5.73 ^C
diacetyl	5.45	5.89 ^A	6.87 ^B	6.73 ^B
2-pentanone	5.80	5.23 ^A	6.95 ^B	6.78 ^B
2-hexanol, 2-ethyl	6.80	5.63 ^A	7.75 ^B	7.08 ^B
hexanal	6.87	5.3 ^A	6.26 ^B	5.16 ^C
2,3-pentanedion	7.04	0 ^A	6.35 ^B	6.54 ^B
1-hexanol	7.80	4.86 ^A	5.55 ^B	4.81 ^A
1-penten-3-ol	7.90	5.00 ^A	4.66 ^B	5.50 ^C
1-pentanol	8.95	4.20 ^A	5.60 ^B	5.41 ^C
3-buten-1-olo, 3-methyl	8.97	3.65 ^A	5.53 ^B	5.64 ^B
acetoin	9.41	6.47 ^A	8.32 ^B	8.12 ^B
2-propanol, 2-methyl	10.00	4.75 ^A	6.58 ^B	5.97 ^C
2-nonanone	10.17	4.55 ^A	7.91 ^B	7.99 ^B
2,4-dimethylheptane	10.66	6.48 ^A	6.25 ^A	6.23 ^A
acetic acid	10.83	6.33 ^A	6.32 ^A	7.99 ^B
benzaldehyde	12.04	5.58 ^A	5.59 ^A	5.49 ^A
3-octanone	12.30	3.80 ^A	5.57 ^B	5.17 ^C
butanoic acid	12.36	5.97 ^A	7.56 ^B	7.58 ^B
2-methyl-butanoic acid	12.50	4.45 ^A	5.74 ^A	5.67 ^A
3-methyl-butanoic acid	12.60	4.50 ^A	5.42 ^B	5.54 ^C
pentanoic acid	13.28	0 ^A	5.55 ^B	4.42 ^C
dimethyl sulfone	14.78	6.2 ^A	5.86 ^B	6.11 ^C
octanoic acid	15.66	5.56 ^A	6.5 ^B	6.48 ^B

Different superscript means significant ($P < 0.05$) differences between mean values of concentration of the same compound in different samples.

Highlighted in grey compounds which concentrations are significantly different ($P < 0.05$) between free and encapsulated cells

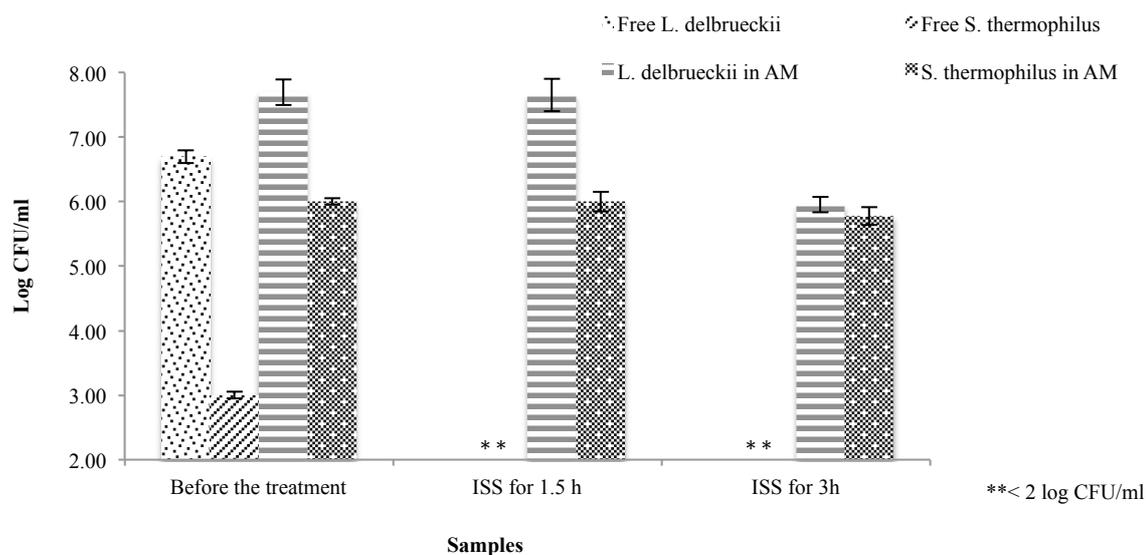


Fig. 7. 5 Viable counts of free and encapsulated untreated *L. delbrueckii* and *S. thermophilus* and after incubation in saliva simulated solution (SSS) for 10 min, gastric simulated solution (GSS) for 2 h and intestinal simulated solution (ISS) for 1.5 h (A) or 3 h (B). The error bars represent standard deviations. AM: matrix microcapsules

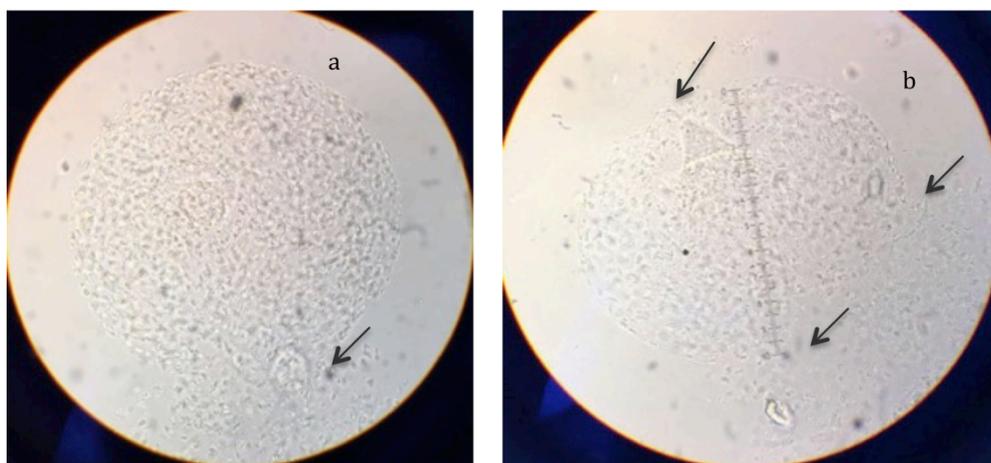


Fig. 7.6 Images (40X) of matrix (AM) microcapsules in yoghurt after exposure to saliva simulated solution (SSS) for 10 min, gastric simulated solution (GSS) for 2 h and intestinal simulated solution (ISS) for 1.5 h (a) or 3 h (b). Arrows indicate points of swelling and resulting cell leakage

7.4 Discussion

In this study an advanced methodology to encapsulate living cells was used. The narrow size and shape distribution, the mouldable thickness of encapsulating matrices along with the smallest achievable microcapsule sizing, aim to guarantee uniform performances (e.g. metabolites exchange, protection toward cells) of a microencapsulated population. Because of the development of novel encapsulating systems, additional investigation is required over the acquired knowledge for the first immobilization systems. In a previous work (De Prisco et al. 2015) vibrational nozzle technology was used to produce matrix microcapsules entrapping *Lactobacillus reuteri* DSM 17938. Accordingly, in this work we were able to produce both matrix

and core shell microcapsules showing excellent performances in terms of entrapment efficiency, shape, size homogeneity and stability. Due to the different conditions of the microencapsulation process and resulting difference in microcapsule morphology, the cell average load for microcapsule was about 5 times more in CAM cells than in MM.

The aspect of cell leaking from microcapsules used during a food fermentation process is poorly investigated and discussed in literature (Corbo et al., 2016). Results on cell leaking from microcapsules showed that in both microcapsule types this phenomenon occurred. Data of Figure 7.3 showed an increase of CFU/ml, probably due to the leaking of cells from the microcapsules. However, in MM it is noticeable just after 3 hours, while in the CAM it starts after 6 hours (Fig. 7. 3). Theoretically, the number of colonies would have to remain unchanged along the time because we assume that each colony rise from one microcapsule (De Prisco et al. 2015). Interestingly, our results showed that despite different kinetics during milk fermentation for the two types of intact microcapsules, cell load values were very similar when analysed after their disruption (Fig. 7. 3).

Microencapsulation of starter culture prolonged the time required for milk acidification and, according to the microcapsule type, it occurred with different kinetics (Fig. 7. 2). The slower acidification operated by encapsulated cells compared to the free one is most likely due to the mass transfer resistance that engaged both the diffusion of nutrients inside the capsules and the release of cell metabolites. This phenomenon also has been observed during milk fermentation by immobilized *Lactococcus lactis* and it has been ascribed to the barrier effect given by the capsules as well as to the cell layer formed on the capsule surface during cell replication (Zhou et al. 1998). Similar findings were reported by Prevost et al. (1985), who aimed to improve acidification performance of starter culture used for the continuous pre-acidification of milk for yoghurt manufacture by entrapping populations of *L. delbrueckii* and *S. thermophilus* in separated capsules. Many conflicting results about increased, unchanged or reduced growth rate of immobilized bacteria used for fermentations have been found (Junter et al. 2002). Decreased or enhanced cell growth were attributed to mass transfer limitation and to the protective effect of the immobilization, respectively (Junter et al. 2002). These conflicting results could be reasonably attributed to the microorganism involved, to the performance and the design of the encapsulating system, as well. Our findings indicate that microcapsule performances strongly depend on capsule morphology, dimension, microorganisms involved and media in which fermentation process is carried out. Using MM capsules, the growing of cells on the outer layer lead to the formation of micro-colonies onto capsule surface (visible in the Panel c of Figure 7.1) from which lactic acid and other metabolites can be easily released. On the other hand the CAM system alginate outer layer likely delayed the diffusion of cell metabolites in the external environment. Another hypothesis for explanation of delayed growth and acidification kinetics is related to the possible accumulation of lactic acid in the capsules that can lead to both retarded medium acidification and cell growing, for the effect of the intra-capsular reduced pH. Champagne et al. (1994) proposed the use of microprobes to investigate the capsular microenvironment but no authors made use of this tool thus far. Considering the similar cell load obtained in MM and CAM at the end of fermentation along with the different acidification kinetics, our findings suggest that size and structure of microcapsules affected more markedly the diffusion of cell metabolites outside the capsules rather than the inflowing of nutrients. However, more specific investigations should be performed to confirm our hypothesis. Further hypothesis that should not be neglected is the possible decline of proto-cooperation between *Streptococcus*

thermophilus and *Lactobacillus delbrueckii* subsp. *bulgaricus*. Recently, Arioli et al. (2016) confirmed that urease activity of *Streptococcus thermophilus* enhanced glycolysis and homolactic fermentation as well as the grow rate of *Lactobacillus bulgaricus*. Hence, the physical barrier of alginate network could reduce the stimulus effect of ammonia on the metabolism of *Lactobacillus bulgaricus* cells.

According to our data, proteolysis operated by encapsulated bacteria was significantly slower than that of free cells (Fig. 7. 4). The nature of LAB proteinase individuated in a cell-envelope proteinase system (Savijoki et al. 2006) can reasonably explain the delayed protein breakdown as consequence of the physical separation created by alginate layer of cell-wall proteinase and milk proteins. The lower proteolysis of samples containing CAM compared to that containing MM is an additional confirmation of this hypothesis. Proteolytic activity of starter culture has a key role in the formation of flavour compounds and in the rheological features of fermented foods. This is especially true in fermented foods like yoghurt in which the natural microbiota is almost completely eliminated by milk pasteurization. On the other hand, amino acids and small peptides obtained by proteolysis of caseins are needed for the regular growth of typical fastidious microorganisms like LAB. Several authors showed positive correlation between proteolytic activity and growth rate in LAB strains (Dandoy et al., 2011; Galia et al., 2016; Mauriello et al. 1998) and this could be a further reason explaining the delayed growth of encapsulated microorganisms with respect to the free one. From another point of view, use of immobilized starter cultures was recommended for the manufacturing of fresh milk-based products (e.g. yoghurt, quark and mozzarella cheese), in which proteolytic phenomena during storage are undesired (Campagne et al. 1994). However, effects arise from encapsulation might be different: Unver et al. (2015) found that entrapment in calcium-alginate improved production and release of extracellular alkaline proteases in *Cryptococcus victoriae*. Despite the increasing number of papers dealing with the use of encapsulated LAB in food fermentation a proper comparison of the findings is hampered by the lack of data about the proteolytic ability of encapsulated microorganisms. Proteolysis of caseins is strictly related to the formation of many volatile compounds implicated in the aromatic notes of fermented milk. However, metabolism of lactose, citrate, lipids and other milk native molecules as well as some metabolic intermediates like pyruvate, is implicated in the aroma formation. In this work, volatile profiles of yoghurt produced by free and encapsulated cells have been characterized in order to elucidate the possible influence of microencapsulation on relevant metabolic traits of starter cultures. As microencapsulation creates a hardly predictable microenvironment, where closer cell-cell and cell-metabolites contact is created and nutrients efflux is also altered, an effect on cell metabolism can be expected. Accordingly, the concentration of 12 out of all 28 compounds detected by GC/MS analysis is significantly different between free and encapsulated cells (Table 7. 2). The number of metabolites is lower than that reported in the literature (Cheng 2010; Ott et al. 1997; Routray and Mishra 2011), but our results are probably caused by the use of skimmed milk. Our results are in agreement with that of other authors that analysed VOCs of yoghurt obtained from skimmed milk (Settachaimongkon et al. 2014). We found that acetone slightly raised only during fermentation with encapsulated cells. Even though acetone originates usually from milk, it could also be produced by the yoghurt bacteria (Carcoba et al. 2000; Georgala et al. 1995) and encapsulation seems to stimulate the biosynthesis of this aromatic compound. Interestingly, we found that encapsulated cells produced both acetic acid and ethanol, while free cells only ethanol in a higher quantity than the encapsulated cells. The production of these substances is associated to the heterolactic

fermentation and their concentrations are inversely correlated. It is known that different pH levels or availability of glucose can cause a shift from homo- to heterolactic fermentation in homofermentative LAB with consequent acetate and other different metabolites production (Borch et al., 1991; Torino et al., 2001) and microenvironment into the capsule can create these conditions. However, some authors reported that ethanol could be produced in yoghurt also by the amino acids breakdown (Cheng 2010) and the reduced proteolysis in yoghurt with encapsulated starter cultures might explain the resulting lower level of ethanol (Table 7.2). It is interesting to underline that Kwak (1995) found a positive correlation between ethanol and off-flavour in yogurt during the storage of 15 days at 10°C. All other substances detected in our study are known volatile flavour compounds identified in plain yoghurt that can affect the aromatic features of the final product (Cheng 2010). As our results showed that microencapsulation affects the cell metabolism, it could be proposed as method to balance the aroma compounds during a food fermentation process.

Matrix chitosan-alginate microcapsules here tested successfully protect both *L. delbrueckii* and *S. thermophilus* cells during storage in yoghurt. Protective effect of alginate-based microcapsules is well documented (De Prisco et al. 2015; Rodriguez et al. 2011; Sousa et al. 2012). Although the protective effect of capsules in acidic environment could be attributed to the barrier effect of calcium-alginate, a more in depth investigation is needed to explain the enhanced cell viability at molecular level. Accurate mimicking of GI conditions is essential for a rigorous prediction not only of cell survival against human GI environment but also of the microcapsule behaviour since the composition of simulating solutions can influence capsule swelling and cell release. Besides the widely reported efficacy of microencapsulation in reducing cell sensitivity to GI transit (Cook et al. 2012; Martín et al. 2015), this work demonstrated that a probiotic strain normally included in pharmaceutical formulations could lose its prerequisite of resistance to gastro-intestinal barriers for the effect of food matrix. In this scenario, the use of microencapsulation might also be considered as a strategy to broaden the number of probiotic strains used in foods.

7.5 Conclusion

Microencapsulation of the probiotic yoghurt starter culture influenced its technological performances in retarding milk acidification and proteolysis and its growth in milk. This effect was more pronounced when bacteria were encapsulated in core-shell type microcapsules (CAM) because of the higher “barrier effect” exerted by the matrix microcapsules (MM), suggesting the key role of microcapsule morphology in determining the behaviour of a cell population. Our results also suggested that encapsulation can induce some modification in metabolic pathways linked to the production of volatile compounds suggesting the use of microencapsulation as a tool for routing food fermentations besides its advantageous effect in cell protection.

Notes:

- This chapter reports the content of paper entitled “Behavior of microencapsulated starter cultures during yoghurt manufacturing” by De Prisco A, van Valenberg H, Fogliano V, Mauriello G. Submitted to Food and Bioprocess technology.

Experiments have been carried out during a period of seven months as guest PhD student at Food Quality and Design group (Dairy division) of Wageningen University (Netherlands).

CHAPTER 8

Proteomics tool for the investigation of encapsulated probiotic *Lactobacillus reuteri* DSM 17938 physiology under simulated gastrointestinal conditions¹

8.1 Introduction

Most of the common commercial probiotic strains belongs to Lactic Acid Bacteria (LAB) group and in particular to *Lactobacillus* genus. Lactobacilli and naturally present as autochthonous bacteria of many food matrices, play a key role in fermentations of dairy and vegetable foods and they are also associated to human and animals intestinal microbioma (Lebeer et al., 2008). These environments are obviously characterized by specific stress factors and in addition, a synergic effect among many detrimental factors can take place influencing bacterial behaviour and performances (Belfiore et al., 2013; Wu et al., 2016). LAB and probiotic strains in particular, developed a plethora of mechanisms at bases of general and specific stress response allowing bacteria adaptation in many different harsh environments (Mills et al., 2011; Hussain et al., 2013). Stress response related pathways are associated to the induction of a large number of genes and to the synthesis of several proteins leading specific or cross-resistance to stress. In this scenario, proteomics tools allow the comprehension of the adaptive stress response through the study of protein expression patterns and changes induced by the different environment as well as of physiological conditions. Proteomic approach has been successfully applied to study microbial dynamics in foods (Soggiu et al., 2016), bacterial adaptation mechanisms to gastric or bile salt related stress (Lee et al., 2008; Alcántara and Zúñiga, 2012), starvation (Hussain et al., 2006) and many other conditions implying bacterial adaptive and resistance phenomena (Di Pasqua et al., 2013; Hussain et al., 2013; De Angelis et al., 2016). Several proteins known as heat shock proteins such as DnaK, DnaJ, GroES, GroEL and ClpB are common to *Lactobacillus* species and have been found involved in the response of a variety of stresses, namely thermic (heat and cold), osmotic, oxidative and acid stress (De Angelis and Gobetti, 2004). Currently, a consistent literature is dedicated to the exploitation of encapsulation as strategy for the improvement of bacterial robustness under technological and human stress conditions. In particular great attention is given to the use of microencapsulation to strengthen probiotics in gastrointestinal (GI) environment (Cook et al., 2012). However, the entire body of works dealing with this topic is at now limited to the development of suitable encapsulation systems for targeted delivery of probiotics in the GI tract and to the assessment of the improved bacterial survival Ding et al., 2009; Nazzaro et al., 2009; Rodriguez et al., 2011; Heidebach et al., 2012; Ramos et al., 2016). The most reasonable hypothesis is that microcapsules act as barrier against the detrimental agents (e.g. enzymes, bile salts) and explain a buffering capacity against gastric pH with the resulting higher cell survival. Besides the increased bacterial viability, no evidence about the possible cellular origins of the

¹ Experiments described in this Chapter are currently ongoing in collaboration with the Laboratory of Microbial Proteomics of the Department of Veterinary Medicine, University of Milan.

high resistance of encapsulated strains are available as well as about what happens to the cell physiology and probiotic metabolic features. Interestingly, proteomics allowed the comprehension of microbial physiology in immobilized/encapsulated strains, in particular for what concerns the different growth rate and metabolites production during fermentations (Junter et al., 2002; 2004). Yet despite, this approach has only been carried out on few microbial species such as *Saccharomyces cerevisiae* or *Pseudomonas fluorescens* and, in the field of probiotics, it is still far from its potentiality. To the extent of our knowledge, only one paper addresses the use of proteomics (by Lab-on-a-chip capillary electrophoresis) to investigate the behaviour of an encapsulated probiotic strain during addition to carrot juice and subsequent incubation in gastric and pancreatic juice (Nazzaro et al., 2009). This study clearly showed different protein profiles in free and encapsulated strains even though proteins with different levels of expression were not identified. The present investigation aims at exploring for the first time on a molecular level the behaviour of chitosan-alginate encapsulated *Lactobacillus reuteri* DSM cell response through a proteomic approach based on 2DE coupled to MALDI-TOF mass spectrometry. In particular, proteome profiles of free and encapsulated *Lactobacillus reuteri* cells after incubation in gastric and intestinal simulated conditions are compared for the identification of differentially expressed proteins and their identification.

8.2 Materials and Methods

8.2.1 Bacterial strain and culture conditions

Lactobacillus reuteri DSM 17938 isolated from Reuterin™ (Noos S.r.l.; BioGaia AB, Stockholm, Sweden) is the probiotic strain used in this set of experiments. Information about its growth and culture conditions are reported in Chapter 3 and 4 of this thesis.

8.2.2 *Lactobacillus reuteri* cells microencapsulation in alginate-based capsules

Lactobacillus reuteri cells were encapsulated in chitosan coated alginate core-shell microcapsules by vibrating technology according to the procedure described in Chapter 4. Briefly, cultures of *Lactobacillus reuteri* in the early stationary phase were harvested by centrifugation at 5200 g for 15 min. Cell pellets were washed twice in sterile 0.01 M phosphate buffer solution (PBS, pH 7.4) and harvested again by centrifugation to be finally suspended in a volume of 1.6% alginate that was equal to the initial volume of cell cultures to obtain a final cell load of 8.70 ± 0.20 or the same volume of PBS to be stored overnight at 4°C and used in the following experiments. Core-shell microcapsules were obtained by a double extrusion with the concentric-nozzle system. Cell-alginate suspension was extruded through the inner nozzle (80 µm) to obtain the cell-loading core of the capsules while the cell free alginate suspension (1.8%) was simultaneously extruded through the external nozzle (200 µm) to produce the shell of microcapsules. Optimal values for the encapsulation of *Lactobacillus reuteri* cells in alginate core-shell microcapsules were previously individuated and are reported in Chapter 4. Hardening of droplets after extrusion was allowed by their incubation in a 0.5 M CaCl₂ solution for 20 min. After the removing of CaCl₂ solution, all microcapsules pellets were washed twice with PBS. Then, they were further coated with a 0.8% chitosan solution by gentle stirring at 200 rpm for 30 min, let to sediment, rinsed twice and finally resuspended in the double of their volume (v/W) of PBS to be stored overnight at 4°C until their use. Three independent experiments of

microencapsulation were carried out and resulting probiotic microcapsules were treated as following described.

8.2.3 Simulated gastrointestinal passage of free and encapsulated bacterial culture

Gastrointestinal conditions were simulated as already reported in Chapters 5 and 6 according to the protocol by Vizoso-Pinto et al. (2006). Briefly, 25 grams of microcapsules or a free cell pellet from 25 ml of *Lactobacillus reuteri* culture were incubated in ratio 1:4 (referred to the volume of samples) in gastric simulating solution (GSS; 5 g/l NaCl, 2.2 g/l KCl, 0.22 g/l CaCl₂ and 1.2 g/l NaHCO₃, 3g/l pepsin, pH 2.5) and incubated at 37°C for 2.0 hours under manually agitation each 20 min. Then, the intestinal simulating solution (ISS; 6.4 g/l NaHCO₃, 0.239 g/l KCl, 1.28 g/l NaCl, 0.5% bile salts and 0.1% pancreatin, pH 7.0) was added in ratio 1:5 to obtain a final 1:10 dilution of the samples that were incubated in ISS at 37°C for 3.0 hours. Viable counts were performed on samples before GI treatment (untreated) and after their incubation in GSS and ISS. For encapsulated *L. reuteri*, cell load was determined after disruption of microcapsules obtained by decimal serial dilutions of the samples in 0.2 M sodium citrate solution (pH 8.0) while samples of free cells were decimally serially diluted in quarter strength Ringer solution (Oxoid). They were further plate counted on MRS agar and incubated 37 °C for 48 h in aerobic conditions. Results are expressed as Log CFU/ml of g for free and encapsulated cells, respectively. Samples were referred as indicated in Tab. 8.1. Samples of untreated free (L0) and encapsulated cells (M0), after 2.0h of incubation in GSS (Lg and Mg) and after the whole treatment under GI condition (Lg+i and Mg+i) were centrifuged at 5500g for the discarding of GI simulated solutions while cell pellets were washed and centrifuged again for following analysis. Three independent replicas of the experiment were carried out.

Tab 8.1 Abbreviations for samples of free and encapsulated *Lactobacillus reuteri* cells before (untreated) and after incubation in simulated gastric (GSS) and intestinal (ISS) solution for 2.0 and 3.0h, respectively

	Untreated	After incubation in GSS	After incubation in GSS+ISS
Free cells	L0	Lg	Lg+i
Encapsulated cells	M0	Mg	Mg+i

8.2.4 Microcapsule dissolution

The total volume (25 g) of recovered microcapsules approximately containing 1×10^{10} cells was suspended in ratio 1:5 in 0.2 M sodium citrate solution. The suspension was held at room temperature for 30 min at constant agitation (150 rpm) to favour the chitosan-alginate matrix dissolution and to obtain a cell suspension free from capsules residues. Thereafter, samples were centrifuged at 5200 g for 10 min and again resuspended in the same volume of sodium citrate, agitated as above described and finally centrifuged for the recovery of cell pellets. Free cell samples after GI simulated passage were treated in the same way.

8.2.5 Preparation and quantification of bacterial protein extracts

About 30 mg of cell pellet from all samples (L0, M0, Lg, Mg, Lg+i and Mg+i) were suspended in 300 μ l of lysis buffer made by 7 M urea (Sigma), 2 M thiourea (Invitrogen) and 2% CHAPS (Sigma) and 0.1 mm Zirconium Silica beads (Biospec products Inc, USA) added in ratio 1:1 (w/v) to the cell pellets. Thereafter, samples were processed along 6 alternated cycles of 1 min in bead beater (Bertin Technologies), 5 min in ice incubation and centrifugation (14000 rpm) for 5 min at 2°C. After all cycles were completed, samples were finally centrifuged (14000 rpm) for 30 min at 2°C. Supernatants were collected to be treated for protein purification. Thus, protein extracts were precipitated in Protein Bind tubes (Eppendorf) following a procedure adapted from Wesse and Fugge (1984) by a first addition of 100% MeOH in ratio 1:2 to the protein supernatant, a subsequent addition, after sample agitation, of Chloroform in ratio 1:1 to the sample and a further addition of MilliQ water in ratio 1:6 to the samples and centrifugation (13000 rpm) for 1 min at room temperature. After removing and discarding the upper layer, 100% MeOH was added again in ratio 1:6 with the initial volume of samples that were firstly agitated on vortex and then centrifuged (13000 rpm) for 2 min at room temperature. Finally, for the recovery of purified protein pellets, supernatants were discarded and protein pellets were air dried.

Afterwards pellets were resuspended in half volume of lysis buffer with respect to the initial volume of the protein extracts and finally stored at -20°C up to following analysis. Protein concentration was determined by Bradford assay. An aliquot of 2 μ l of purified protein extracts were let incubated at room temperature for 5 min in 800 μ l of MilliQ water and 200 μ l of Protein Assay Stain (Biorad). Optical density was measured at 595 nm using a spectrophotometer (Gene Quant 100, GE Healthcare) and protein concentration was determined against Bovine Serum Albumin (BSA, Thermo Scientific) used as standard. The total protein extraction and purification was performed for the samples obtained from the three replicas of the experiments of GI simulated transit, both for free and encapsulated cells. The protein solutions were finally stored at -20°C and used in subsequent analyses.

8.2.6 2D-PAGE

2D Electrophoresis was performed as described by Piras et al. (2015). The first-dimension isoelectric focusing (IEF) of proteins was run by using immobilized pH gradient (IPG) polyacrilamide gel strips (GE Healthcare, 7 cm, pH 4.0-7.0) and Protean IEF Cell (Bio Rad). Prior to IEF, 100 μ g of purified proteins from each sample were dissolved in a solution of 30 mM DTT, 0.5% w/v Ampholine (pH 3.5-10.0), 1% w/v bromophenol blue and lysis buffer to a final volume of 126 μ l. Samples were carefully distributed onto IPG strips that were actively rehydrated at 50 V and 20°C for 18 h. For protein focusing, the voltage was gradually increased according to the following protocol; 100 V (4 h), 250 V (2 h), 4000 V (5 h), 4000 V until the cumulative voltage reached 60 kVh, then it was decreased to 250 V. A limit of 50 μ A per gel strip was set. Water imbibed paper wicks were previously placed between cathode, anode and gel strips for preventing their burning. After IEF, proteins on each strip were reduced for 15 min in 5 ml of Equilibration buffer (6 M Urea, 2% w/v SDS, 50 mM Tris-HCl buffer and 20% v/v Glycerol in MilliQ water, pH 8.8) with 1% w/v DTT, and then, after a gentle removing of previous solution, they were alkylated in 5 ml of Equilibration buffer with 2.5% w/v of Iodoacetamide for 15 min in dark incubation. Each IPG strip was washed in 1X

running buffer (1% w/v SDS, 250 mM Tris-HCl, 1920 mM Glycine in MilliQ water, pH 8.8) and loaded onto 12% w/v polyacrylamide resolving gel (10 ml; 4 ml of 30% Degassed Acrylamide/Bis, 3.4 ml of MilliQ water, 2.5 ml of 1.5M Tris-HCl, 0.1 ml of 10% SDS solution, 5 µl of Temed and 50 µl of ammonium persulfate solution) along with the protein ladder (Precision Plus Protein All Blue Standard, Biorad). IPG strips were sealed on top of the SDS-PAGE gel using 0.5% agarose. Second dimension was carried out in Mini-Protean Tetra system (Bio Rad).

SDS-PAGE was run in Tris-glycine-SDS buffer system (25mM Tris, 192mM glycine and 0.1% SDS) at 8 mA per gel for 15 min until the bromophenol blue entered the resolving gel and than 16 mA per gel were applied until the dye front reached the bottom edge of the gel. Gels were washed three times in 100 ml of deionised water for 5 min under gentle agitation and then left in overnight incubation in 100 ml of preheated Brilliant Blue Coomassie G-250 solution (60 mg/L Brilliant Blue Coomassie, 3ml/L HCl) for staining.

8.2.7 Image analysis

2-DE protein patterns were developed as digitalized images using a flatbed scanner (ImageScanner III, GE Healthcare, Uppsala). Before scanning, gels were washed for 20 s in 70% v/v ethanol and then for 2 min in 100 ml of deionised water. Variations in protein expression revealed between free and encapsulated *L. reuteri* cells after simulated gastrointestinal passage were analysed using the Progenesis SameSpots software (Nonlinear Dynamics, UK), Version 4.6. The module for 2DE gel analysis was used for images aligning, background removal and detection, normalization and matching of the spots.

Since only two out of the three replicas of the samples have been analysed up to now, statistical analysis will be completed using the Progenesis SameSpots Stats module on the log-normalized volumes for all spots once all the replicas will be available. Stats module performs automatically a One-way ANOVA on each spot to evaluate the *P*-value between different groups. *P*-values under 0.05 will be considered statistically significant.

8.2.8 Protein identification by MALDI-TOF MS

Proteins with significant different ($P < 0.05$) levels of expression will be identified by Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) after spots reviewing and selection for manually excision from 2 DE gels.

8.3 Results

8.3.1 Viability of free and encapsulated *Lactobacillus reuteri* after GI simulated passage

The behaviour of free and microencapsulated *Lactobacillus reuteri* DSM 17938 cells in coping with the stress from GI environment is described in Fig. 8.1.

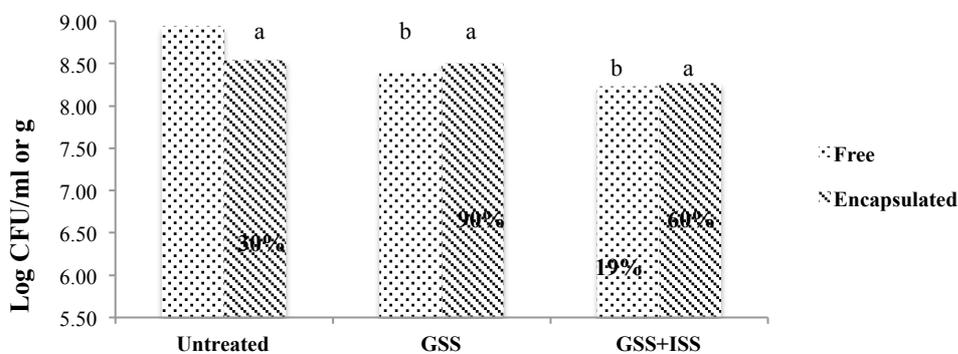


Fig. 8.1 Viable counts and survival percentages of free and microencapsulated *L. reuteri* cells before (untreated) and after incubation in gastric (GSS) and gastric plus intestinal simulated solutions (GSS+ISS). Data are expressed as mean values of three independent replicas of the experiment and standard deviations were always <0.1 . Different letters labelling bar graphs of the sample categories indicate that mean values are significantly different ($P \leq 0.05$) as determined by t-test.

Lactobacillus reuteri had different performances of survival under gastrointestinal simulated conditions when treated in free and microencapsulated form. When treated in free form, *L. reuteri* population showed a notable resistance both in GSS and in the entire GI passage. However, after its incubation in GSS, a ($P \leq 0.05$) significant reduction of the 70% of the population was recorded against a reduction of only 10% in microencapsulated population. The survival level of free and microencapsulated *Lactobacillus reuteri* population dropped to the 19 and 60% after the subsequent incubation in ISS, respectively. Interestingly, this finding indicated an overall higher resistance of microencapsulated cells after incubation in GSS and ISS but a higher resistance of free population in its passage from gastric to intestinal incubation, as demonstrated by a reduction of about the 10% of population against a reduction of the 30% of microencapsulated population.

8.3.2 Protein patterns of free and encapsulated *Lactobacillus reuteri* in response to GI simulated passage

Proteomics analysis of *Lactobacillus reuteri* treated in free and encapsulated form under GI conditions was performed in order to investigate at molecular level the stress-response of the probiotic strain when conveyed to intestinal tract in free and microencapsulated form. Protein analysis by 2D Electrophoresis of untreated microencapsulated cells (M0) and of microencapsulated cells after incubation gastric simulated solutions (Mg) was partially unsuccessful by the probable residue of microcapsules that interfered with IEF of the samples (data not showed). Consequently, 2DE analysis of untreated *Lactobacillus reuteri* in free form (L0), here considered as control, and *Lactobacillus reuteri* after the complete GI passage in free (Lg+i) and microencapsulated form (Mg+i) were performed and compared. Only two out of the three replicas of the experiments have been analysed until now and thus, a statistical

analysis of the protein patterns in the three samples has not been already accomplished. Representative 2 DE gel profiles of samples L0, Lg+i and Mg+i are represented in Figs. 8.2 a, b and c. Even though a statistical analysis has not been already performed for a complete spot comparison and determination of significant differences among protein patterns, some differences in the presence of some spots and their relative expression levels are appreciable in Fig. 8.2. This is also confirmed by a preliminary and partial analysis carried out with Progenesis SameSpots that indicates possible difference in the relative presence/absence and in different expression levels of several proteins among the three samples (Fig. 8.3). Accordingly, panel a of Fig. 8.3 shows the expression of a high molecular weight protein (and the ones next to it) in samples L0 and that it was down-regulated in both free and encapsulated cells after exposure to GI conditions. Differently, panel b of Fig. 8.3 shows an example of an overexpressed protein in samples Mg+i with respect to L0 and Lg+i. Opposite situation is depicted in panel c that shows the overexpression of a protein in the treated free cell with the respect to the control and to the microencapsulated counterparts.

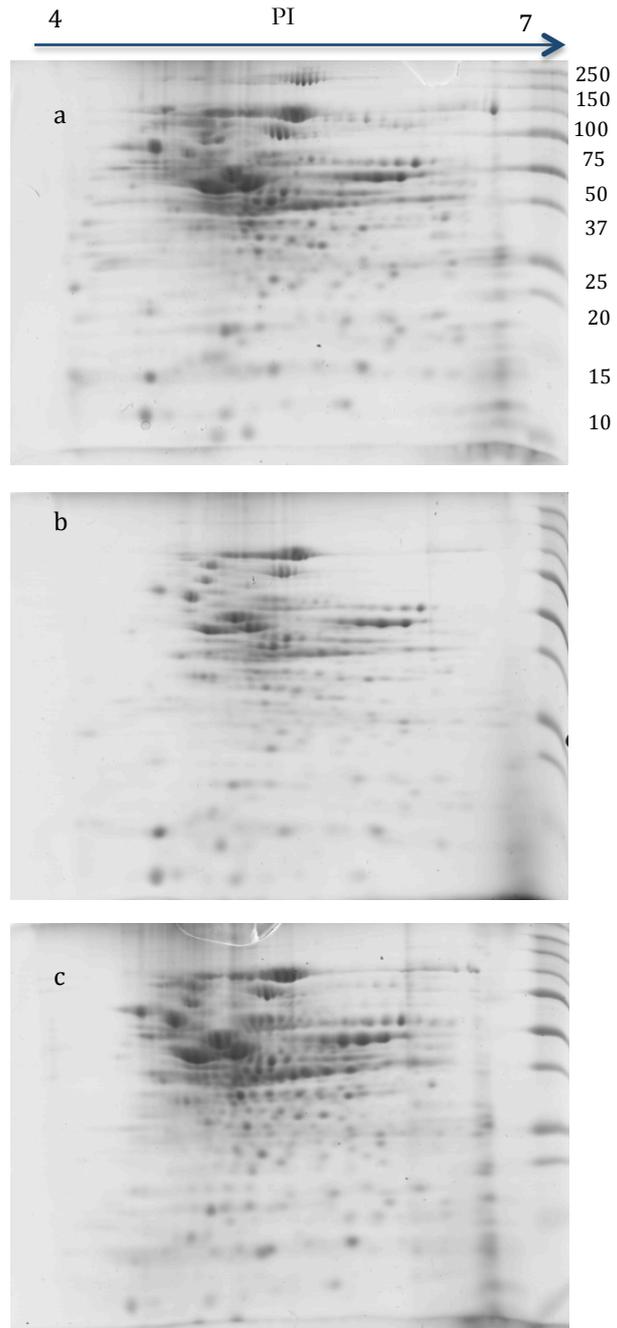
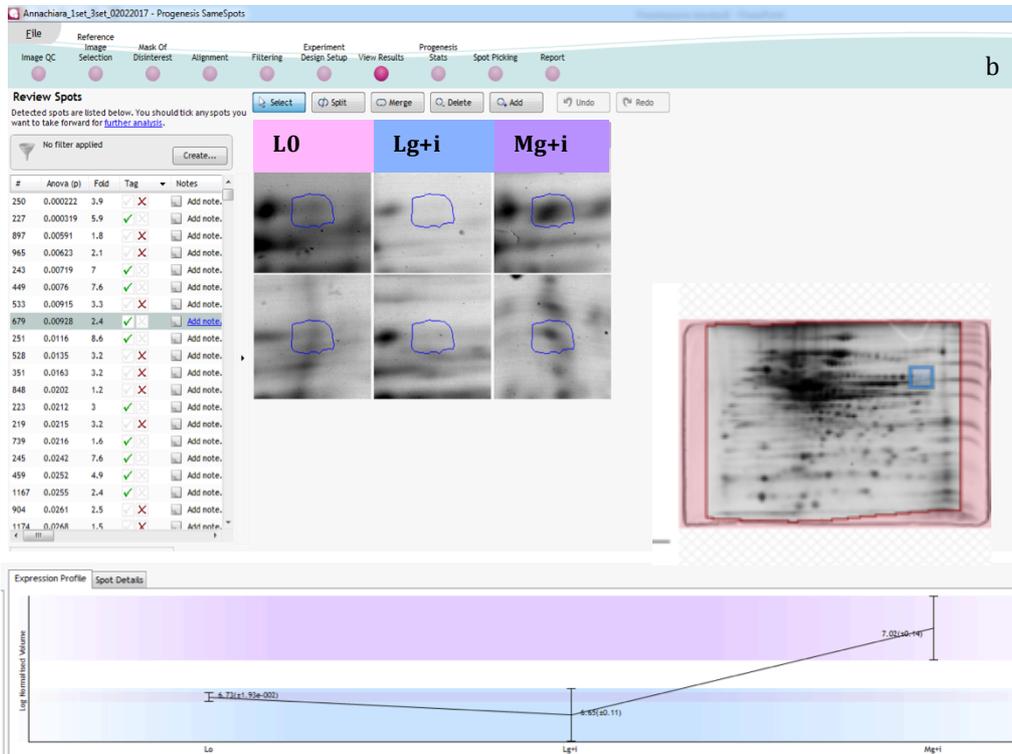
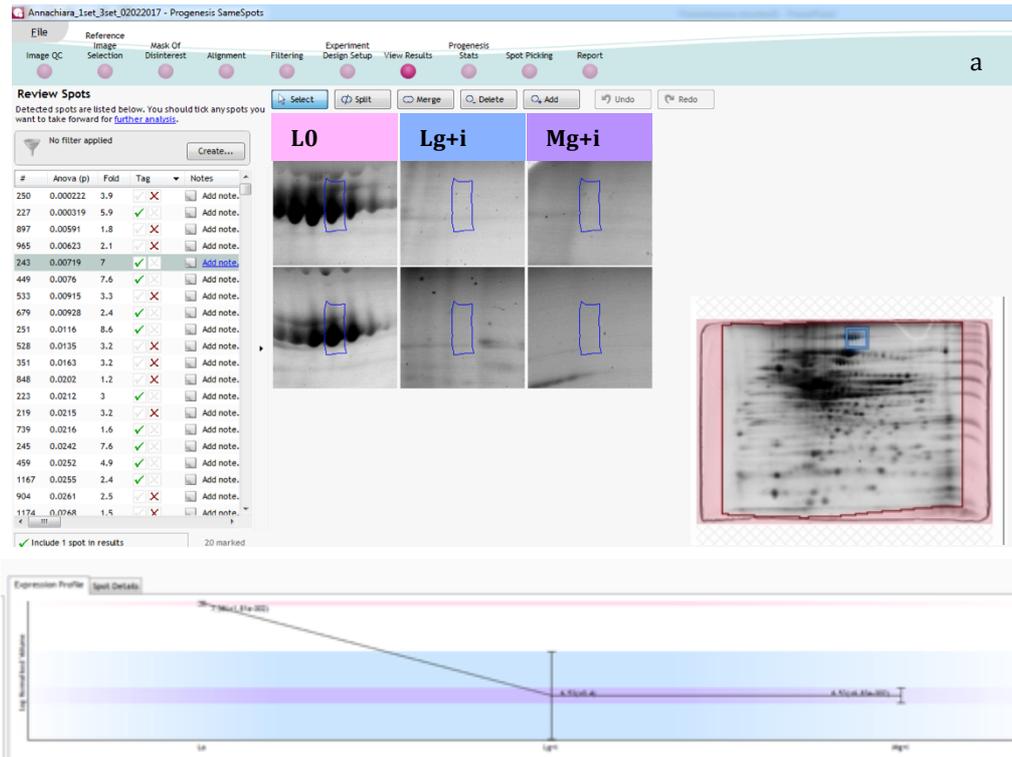


Fig 8.2 Brilliant Blue Coomassie-stained 2DE gels of total cell proteins extracted from free untreated *L. reuteri* DSM17938 (L0, panel a) and treated under gastrointestinal simulated passage in free (Lg+i, panel b) and encapsulated form (Mg+i, panel c). The figure shows one representative gel for each sample.



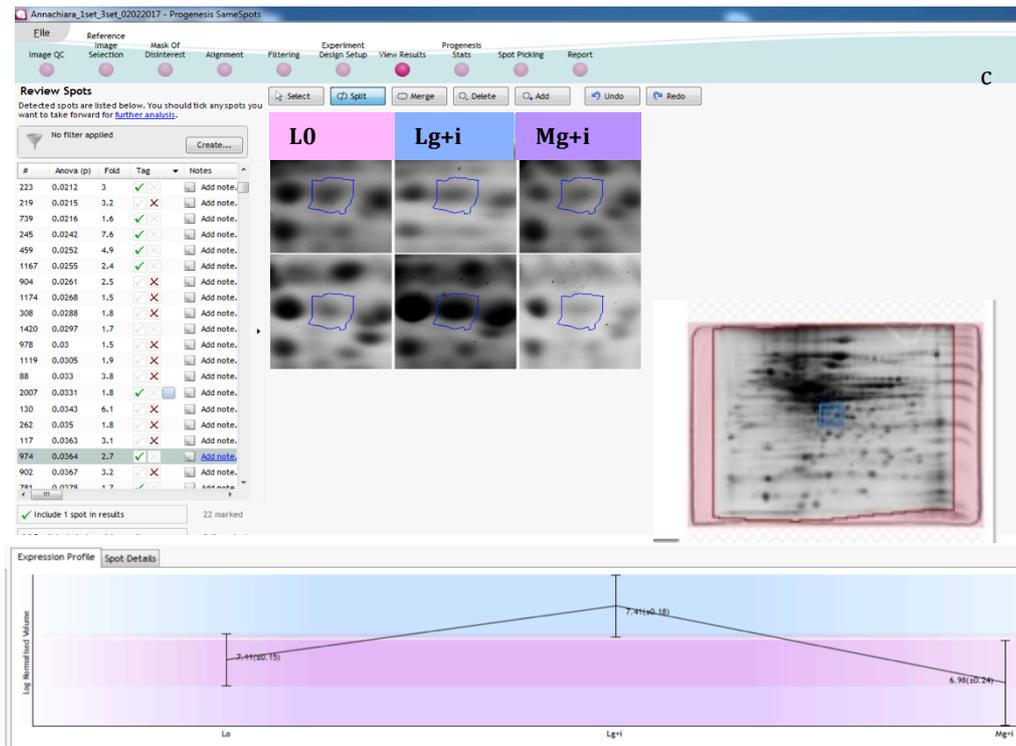


Fig. 8.3 Spot identification by Progenesis SameSpots image analysis in protein profiles of untreated free *Lactobacillus reuteri* DSM17938 cells (L0) and of free (Lg+i, panel b) and encapsulated *L. reuteri* (Mg+i, panel c) after incubation in gastrointestinal simulated conditions. 2DE protein pattern of L0 was used as reference gel.

8.4 Discussion

Driven by consistent growth in the body of knowledge dealing with the use of microencapsulation to endure probiotic bacteria, this group of experiments aim for the first time at the exploitation of the molecular bases of the increased microencapsulated probiotic viability as well as of cell physiology under gastrointestinal conditions. At this purpose, a well-known probiotic strain has been treated in simulated gastric and intestinal conditions in free and encapsulated form for the analysis and comparison of protein patterns by Two Dimensional Gel Electrophoresis. In accomplishment with the gastrointestinal resistance as important prerequisite for the selection of probiotics, free cells of *Lactobacillus reuteri* DSM 17938 exhibited a notable level of viability when tested against human barriers such as low pH, digestive enzymes and bile salts (Fig. 8.1). Nevertheless, the viability of *L. reuteri* population was further improved by the microencapsulation as demonstrated by the higher level of survived cells recovered after the GI passage (Fig. 8.1). However, in order to fulfil their beneficial action, probiotics should be able to sense the environment of the GI tract and to adhere to the intestinal mucosa (Siciliano and Mazzeo, 2012). In this perspective, in spite of the large application of microencapsulation on probiotics, what happen to the physiology of probiotic bacteria after to instauration of new microenvironment given by the capsules is still fully unexplored. The most probable reasons at the bases of microcapsule preservation ability are recognized in their barrier and buffering effect that play an essential role to limit the contact of

probiotic cells with the stress factors (Cook et al., 2013; Nualkaekul et al., 2013). In addition, with this set of experiments we aim to investigate whether the enhanced cell survival can be also attributed to other important factors namely, the new physic-chemical environment within the capsules where the cells and their metabolites are closely related and whether they can influence cell physiology having a role in cell stress resistance. At this purpose, the use of proteomics tool is increasing providing important information about the changes of protein expression patterns in bacteria induced by specific and physiological and environmental conditions. Our preliminary results indicated that some proteins were up- or down- regulated in microencapsulated *Lactobacillus reuteri* during simulated gastrointestinal transit (Fig.8.3). Unfortunately, the investigation of microencapsulated cells recovered at the beginning (M0) and after GSS exposure (Mg) has been hindered by probable alginate and chitosan residues that interfered with IEF phase. Differently, the analysis of microencapsulated samples after gastric and intestinal incubation was allowed by the dissolving action of the intestinal reproduced conditions on alginate-chitosan complex, as also reported in Chapter 7, necessary to permit cell release of probiotics in the target site. Future identification will reveal which functional protein categories (e.g. proteins involved in signal transduction, transcription and translation and general stress; cell-envelope proteins) might be involved in the different stress response of encapsulated bacteria as well as how microencapsulation can influence probiotic related pathways. In the same time, this research will also provide relevant information on the global protein profile of a probiotic *Lactobacillus reuteri* strain following the subsequent exposure to gastric and intestinal conditions since, to the extent of our knowledge, only the response to low pH (Lee et al., 2008a) and bile salts (Lee et al., 2008b; Yanina Bustos et al., 2015) have been separately investigated through proteomic analysis. In spite of the potential application of proteomics tools on microencapsulated microorganisms, few investigations have been carried out in this field and they generally not concern probiotics. Accordingly, Westman et al. (2012) studied the proteomic profile of *Saccharomyces cerevisiae* by 2-D DIGE and quantitative nLCMS/MS, shed a light on the mechanisms sustaining the increased thermoresistance and ethanol production and the decreased biomass yields of alginate-encapsulated yeasts compared to the free cells. These phenomena were mainly attributed to stricter anaerobic and nutrient starvation conditions of the encapsulated cells leading to an up-regulation of proteins related to trehalose and glycogen (membrane protectors) synthesis and utilization, alcohol dehydrogenases and many stress-related proteins along with the down-regulation of the proteins linked to protein synthesis. An evidence of different protein profiles expressed by a probiotic strain of *Lactobacillus acidophilus* added to carrot juice after GI simulated passage has been reported by Nazzaro et al., (2008) but no data about protein identification are available. Also the bacterial cross-talk by *quorum sensing* (QS) has been found implied in regulation of cell behaviour (Di Cagno et al., 2011). Interestingly, Gao et al. (2016) has recently discovered an improved stress resistance of *Escherichia coli* cells when encapsulated in chitosan-alginate matrices, mediated by an up-regulation of luxS/AI-2 system. With their findings, they demonstrated the encapsulated cells had a stronger *quorum sensing* capacity since they were aggregated and confined in microcapsules promoting cell-cell-signal interactions and QS related molecules accumulation and reducing cell-cell communication distance (Gao et al., 2016). These findings provide reasonable evidences to let us consider a deep influence of new environment within the capsules on cell physiology.

8.4 Conclusion

The topic of the present work is the exploitation of the physiology of microencapsulated cells by proteomics approach. Even though the experiments are still ongoing, preliminary results showed different levels of expression of some proteins indicating differential stress response in microencapsulated bacteria with respect to their free counterparts. Future findings could open a new area of research looking forward the elucidation of the influence of microcapsules on probiotic cell behaviour in many different stress conditions. For example, the reason behind the higher probiotic viability during long-term storage or the increased bacteriocin production from certain probiotic cells could be fully addressed. At this purpose, the refinement of the procedure of microcapsules dissolving is of paramount importance for the recovery of cell pellets suitable for genomic and post-genomic analysis. In this perspective, a comprehensive study of encapsulated microorganisms behaviour at cellular level will provide essential information about the metabolic state and long-term behaviour over the general information of their increased viability. Furthermore, it also could drive the development of new encapsulating systems thought on the base of the interaction between the encapsulating matrices and target probiotics.

Notes:

Experiments described in this Chapter are currently ongoing in collaboration with Dr. Paola Roncada, of Istituto Spallanzani and Dr. Alessio Soggiu, Dr. Cristian Piras and Dr. Isabella Alloggio of the Laboratory of Microbial Proteomics- Department of Veterinary Medicine, University of Milan.

CHAPTER 9

General discussion

Microencapsulation technology has become an effective strategy for enhancing the resistance and functional features of microorganisms. A consistent body of knowledge providing evidence on the versatility of this technology has accumulated over the years. Successful application of the technology both at laboratory-scale and industrial process has enabled its adaption for the encapsulation of many classes of bacteria, ranging from those used as starter cultures and biocatalysts (Rokka and Rantamäki, 2010; Westman et al., 2012; Adbel-Rahman et al., 2013), to probiotics, which are considered an important promoter of human health (Douglas and Sanders, 2008; Del Piano et al., 2011; Mills et al., 2011). Over the years, research has shown that microencapsulation of cells used as starter cultures or biocatalyst improves their fermentative characteristics, including their metabolite production, robustness and operational stability of the fermentation system. Furthermore, from an industrial point of view, cell encapsulation has become one of the most useful methods for increasing cell concentrations in fermentors, enabling their re-utilization and continuous fermentation at high dilution rates (Adbel-Rahman et al., 2013). Among other applications, microencapsulation was proved to be a successful tool in the sector of probiotic microorganisms for the enhancement of their ability to reach the intestine in higher amounts and for the improvement of their robustness during their production and utilization as food ingredient or supplement. Probiotics are defined as “live microorganism, which, when administered in adequate amounts, are able to confer a beneficial physiological effect on the host” (Expert Committee FAO/WHO, 2001). Their health benefits are worldwide recognized and include anti-mutagenic, anti-carcinogenic and anti-infection properties, immune system stimulation, reduction of serum cholesterol and lactose intolerance and nutritional enhancement raising a high interest from probiotic industry in broadening their use in foods (Dong et al., 2013). However, while probiotic food products comprise between 60 and 70% of the whole functional food market (Tripathi and Giri, 2014), the probiotic food sector is almost exclusively leads from represented by fermented and non-fermented dairy products (De Prisco and Mauriello, 2016).). Nevertheless, the health benefits attributed to probiotics has created impetus to extend their application in other categories of foods. In this optic, microencapsulation is used as valid strategy for the improvement of probiotic viability against technological and food-related hurdles. In this thesis, the potential of microencapsulation technology to improve probiotic viability against technological and food-related hurdles was investigated. In building the rationale for the studies reported in this thesis, a review on the application of microencapsulation was conducted until September 2015. The review revealed a notable number of new probiotic food carriers developed at research level (Fig. 9.1). In addition, the review provided useful data on the distribution of new probiotic products in different food categories obtained by the addition of encapsulated probiotics.

As previously discussed in this thesis, despite the relevant improvements toward the useful application of microencapsulation technology to probiotic microorganisms, some challenges still need to be addressed for full industrial adoption of cell microcapsules. In these regards, although some enterprises are involved in the production of probiotic microcapsules and many patents have been issued (Probiocap[®]; Duaolac[®]; Intelicaps[®]) to the extent of our knowledge, no products carrying microencapsulated probiotic bacteria is currently present on the market.

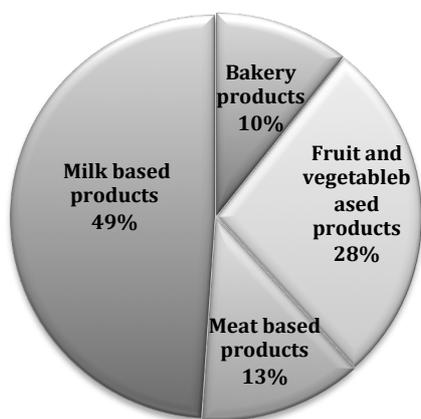


Fig 9.1 Percentage distribution in the current literature of papers dealing with probiotic microcapsules application in different food categories. Image by De Prisco and Mauriello (2016)

Prior to conducting studies reported in this thesis, we did not have any experience with procedure of encapsulation of living cells by vibrating nozzle technology implemented on the device Encapsulator B-395 Pro (BÜCHI Labortechnik). Our previous experiences with encapsulation was derived from spray-drying and emulsion technologies for which we registered poor viability of some lactobacilli strains (by spray-drying) and recovered of microparticles which were non homogeneous in shape and dimension distribution together aswell as low entrapment efficiency (emulsion). By introducing this recently developed extrusion-based technology we achieved promising results in terms of encapsulation efficiency, microorganism viability and morphological and functional properties of microcapsules. These aspects were first addressed in Chapter 2 of this thesis, providing the impact of the encapsulation procedure on cell viability and the effect of encapsulating parameters on the quality of resulting microcapsule population. In detail, cells of *Lactobacillus reuteri* DSM 17938, used as probiotic “model”, were successfully encapsulated, showing a matrix type morphology with a homogeneous spherical shape and diameter. The optimization of the encapsulating parameters is considered fundamental for the recovering of microcapsules with the best properties for food application. Indeed, even though some general indications about the use of the Encapsulator are provided by the manufacturer (Buchi Lab.), only an accurate study of encapsulating parameters allows us to obtain microcapsules with the desired final morphological properties. Our opinion was recently confirmed by Yeung et al. (2016), who reported that encapsulation of strains of *Lactococcus lactis* by adopting the manufacturer’s standard operating conditions led to the production of non-spherical capsules with an irregular dimension, with a diameter ranging from 7 to 480 μm . Beside morphology, experiments reported in Chapter 2 showed an efficient protective feature of microcapsules on cells during freeze-drying and storage; and cells exhibited a high survival

capacity as measured by preliminary experiments of GI simulated passage. These results confirm previous findings, in which of the twelve different microorganisms (including yeasts and bifidobacteria) being encapsulated by vibrating technology, none of them exhibited significant loss of viability. Up to now, at least twelve different microorganisms (including yeasts and bifidobacteria) have been efficiently encapsulated by vibrating technology and none of them exhibited significant loss of viability. Finally, based on the extensive literature and our personal experience, the recovery of high cell viability and the obtainment of microcapsule population with good morphological and functional features are of paramount importance in the field of living cell encapsulation. In this optic, we can consider that the vibrating nozzle technology met all these requirements since it yielded spherical microcapsules with a narrow size distribution and a notable cell viability raising the 100%.

Following observation of good properties of basic alginate matrix microcapsules, the latter were tailored as described in *Chapter 3*, through the inclusion of xanthan gum in their formulation and the evolution in the core-shell morphology. It has to be underlined that, even though the term core-shell is very often referred to matrix microcapsules to which a second layer is applied during a further coating step, the formulation of the real core-shell microcapsule morphology is poorly reported. Alginate blended with xanthan resulted in microcapsules with a likely higher porosity as suggested by our results. Indeed, although a morphological analysis of the alginate-xanthan microcapsules (AX) is required to confirm our hypothesis, we found a weaker ability to protect cells during thermal treatments than microcapsule obtained by only alginate (AM), suggesting that AX were more permeable to hot milk. The inclusion of xanthan also led to the obtainment of core-shell microcapsules with non-perfectly moulded core, as demonstrated by the presence of some cells present in the alginate layer. On the other side, the presence of xanthan prolonged *Lactobacillus reuteri* cell viability during the storage suggesting its advantageous prebiotic effect toward this probiotic strain. Further studies can be carried out to confirm this effect and to exploit alternative use of xanthan gum for *Lactobacillus reuteri* cell protection. Chitosan coated alginate core shell microcapsules behaved as the best microcapsule system in terms of cell protection during thermal treatments, confirming the hypothesis that bigger the capsules size the higher the protection exerted. Yet despite, we believe that through this core-shell microcapsule system the protective effect and particle size are rightly since these microcapsules had an acceptable diameter of about 310 μm . Even though we developed a promising microcapsule system able to exert a thermal protection by using only two relatively cheap encapsulating agents (i.e. alginate and chitosan), we conclude that the exploration of hydrophobic coating agents (e.g. stearic acid) should be addressed to optimize the protection features of the capsules.

The gastro-protective effect of probiotic strains can be considered the most important achievement of microencapsulation to the extent that this technique has been regarded as the future of probiotic human delivery (Vidhyalakshmi et al., 2009; Hernández et al., 2010; Del Piano et al., 2012). Although the gastrointestinal resistance is one of the most important requirements for probiotic selection, it cannot be assumed that all commercial probiotic strains show this feature. Indeed, the storage time or the interaction with food matrices can affect the bacterial stress resistance. As already discussed, microencapsulation showed to increase the stability of probiotics during inclusion and storage in food as well as during simulated transit through the GI tract. With the results reported in *Chapter 5* we confirmed this

evidence but we also demonstrate that microencapsulation can confer the GI resistance to new isolated and potentially probiotic strains selected for interesting and important technological features. Also in this set of experiment, chitosan coated alginate core-shell microcapsules exhibited the best protective performances. In our opinion, more attention has to be given to an accurate simulation of gastrointestinal environment in its composition and physiology. In this regard, a better simulation of human digestive tract including the intestinal microbiota can be achieved through the use of computer-controlled human GI models (e.g. Simulator of the Human Intestinal Microbial Ecosystem, SHIME) in order to project this research at a higher level of investigation.

The formulation of unconventional probiotic foods could represent an attractive form of consumption for humans resulting in an increased assumption of these beneficial microbes. Similarly, the disposability of a variety of probiotic foods already compliant with local food habits can support consumers in unconsciously growing consumption of probiotics. In this framework we use three different food matrices such as Water-Buffalo Mozzarella cheese, almond milk syrup and blueberry juice to test their “probiotication”, as described in *Chapter 6*. Through the accurate selection of the most suitable microcapsule system for each of the food system tested, we obtaining promising result about their potential functionalization with probiotic cells. Future research has to address i) the monitoring of cell stability along capsule production and storage and their utilization during the entire food production and ii) the sensorial analysis of product enriched with probiotic microcapsules.

Since most of the investigations are focused on the addition of microencapsulated probiotic to foods in a dose (10^6 - 10^7 CFU/ ml or g) required to assure the probiotic effect, these bacteria are generally not required to grow in a food matrix. As in consequence, the impact of microcapsules and the new microenvironment on primary cell metabolism is few investigated. Because we believe that probiotic bacteria can also be included in the production of fermented food products, we carried out the experiments described in *Chapter 7*. We encapsulated a mixed yoghurt starter culture prepared with a probiotic strain of *Lactobacillus delbrueckii* to test its ability to ferment milk and to produce volatile compounds when in encapsulated form. This set of experiments was also thought to investigate the permeability of chitosan-coated matrix and core-shell microcapsules in exchanging nutrients and metabolites with external environment. As expected we found that, because of the larger diameter and the presence of the alginate shell, core-shell microcapsules limited the efflux of nutrients/metabolites with higher extent than the matrix microcapsules. However, interestingly, a similar grow rate was found both in matrix and in core-shell microcapsules. So, in this sense we can indicate the core-shell system more suitable for fermentations processes where a high cell density alongside the separation of cells from fermentative media wants to be achieved. It can be assumed that the entrapment in matrix microcapsules let the starter culture to behave as slow-acidifying cultures requiring longer time to ferment milk. On the other hand encapsulated strains were protected during the storage, prolonging yoghurt shelf life, and during yoghurt simulated transit where free cells of *L. delbrueckii* showed poor resistance even if probiotic. Another interesting finding is the evidence that, although the volatile patterns of yoghurt fermented by free and encapsulated strains were similar for what concerned some of the most important yoghurt aroma compounds, they differ for ethanol, acetone and other compounds content. This was likely imputable to the new conditions of the microenvironment suggesting the microencapsulation as a tool to influence aroma formation during fermentation process.

The great potential of probiotic cells is recognized and appreciated in an extremely wide range of areas related to human health. Nevertheless, for optimum action of these probiotic cells, it is essential that they are provided with appropriate conditions for growth and metabolism, and that they are protected from harsh environmental conditions. As previously discussed, the potential of suitable microcapsules to protect probiotics is uncontroversial but the only outcome that has so far been realised following many years of intensive research, is the protection of viability of probiotic. Apart from a few pieces of evidence about the ability of probiotics to produce bacteriocins after their microencapsulation, information on metabolism of encapsulated probiotic cells is hardly available. Similarly, to the best of our knowledge, evidence at a molecular level about stress-response of encapsulated probiotics have not yet been adduced. In this context therefore, experiments described in *Chapter 9* of this thesis produced at very preliminary level results which showed different levels of expression of some proteins. The identification of these proteins will be performed in order to fully understand which probiotic- and stress response-related proteins are potentially influenced by the microenvironment created by the capsule. Finally, the present research aims at introduce a new approach based on the application of proteomic tool to study the behaviour of microencapsulated probiotics, with the view of understanding physiology of encapsulated probiotic cells.

Recommendations

In the future, new food sources of probiotics will likely play a significant role in the human health. However, to fully realize the potential application of encapsulated probiotics for the broadening and quality improvement of probiotic food product, a range of multidisciplinary actions should be pursued as part of a cooperative model to address the following points:

- evaluation and optimisation of drying processes to allow the marketing of dried microcapsules;
- understanding the relationship between capsule material, dimension and their nutrient/metabolite diffusion kinetic which is essential to allow their use in food matrixes with specific processing and storage conditions;
- comprehension, at molecular level, of the effect exerted by the capsule microenvironment on cell physiology in the different processing and utilization conditions;
- advancement of sensorial impact of probiotic capsules in real food systems;
- development of a clear and comprehensive shared guidelines and legislative standards at international level that would lead towards the full deployment microencapsulated probiotics and to boost their introduction in the food market.

But to fully realize the potential of microencapsulation in contributing at the development of new food formula there is the need to create a solid bridge between academia and both small and large enterprise to ensure a fruitful exchange of scientific and technological knowledge.

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About the Author

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Annachiara De Prisco was born the 19th of January 1986 and lives in Salerno, Italy. She obtained her high school diploma at the Classical Lyceum in Nocera (Salerno). She started her Bachelor in Food Science and Technology at University of Naples, Federico II, which she concluded with a thesis entitled “Apoptosis in bacteria”. At the same University, she obtained her Master in Food Science and Nutrition with a thesis on the “Microencapsulation of essential oils: the case of carvacrol”.

During her studies, she was employed for three months at Europa Conserve, Sant’Antonio Abate, 80059 (Na), Italy, a small company involved in the production of tomato derivatives with the mention on quality control and HACCP. Then, she was employed for four years for the quality assurance and quality system management according to the standard UNI EN ISO 9001:2008 and IFS GSF at Jomi S.p.a., Via delle Industrie, 95, 80067-Bellizzi (Sa) and Via Roma, 50- Langhirano (Pr), Italy, a big Enterprise in the sector of production and packing in modified atmosphere of meat derivatives. She concluded her career in the company when she was employed as research fellow at University of Naples, Federico II. Dept. of Agricultural Sciences, Portici (Na). Division of Microbiology, for the development of novel encapsulated ingredients, in particular antimicrobial substances (bacteriocins, essential oil) and the evaluation the performance of encapsulated bioactives within the program PON Research and Competitiveness 2007-2013, project title : “Encapsulation of active ingredients for improving the quality and food safety”. This was followed by the PhD research project at the Microbiology division of the Dept. of Agricultural Sciences at University of Naples, Federico II. During the three years of her PhD she visited for three times the Ugandan University of Gulu where she met Duncan Ongen, Dean of Faculty of Agriculture and Environment and co-tutor of her PhD thesis. During the longer stay she supported her PhD tutor, Prof. Gianluigi Mauriello in the start up of a laboratory for molecular microbiology at Gulu University and in training for students and future trainers in molecular microbiology techniques. She also attended a training School in Strasbourg University on Microencapsulation organized by Strasbourg University and Bioencapsulation Research Group.

She conducted part of this research in at the Department of Agrotechnology and Food Sciences, Food Quality and Design Group of Wageningen University, the Netherlands.

Outcomes of this PhD project have been presented in this thesis, entitled “Effect of microencapsulation on functional and technological features of probiotic and starter cultures”

I want with all my heart and my sincerity express my thanks for my PhD period.

Thanks to my parents, my colleagues, my friend and colleague Diamante, thanks to my tutor, thanks for many opportunities to challenge myself, for the new friends I met, for travelling, for working in the best field ever, the research.

I will never forget these three years, the most incredible ones of my life.

Publications

- De Prisco A., van Valemberg H.J.F., Fogliano V., Mauriello G. Behaviour of microencapsulated starter culture during yoghurt manufacturing. In submission to Applied Microbiology and Biotechnology.
- Mauriello G., De Prisco A., Di Prisco G., La Storia A., Caprio E. Microbial characterization of bee pollen from the Vesuvius area collected by using three different traps. Submitted to PLOS ONE.
- De Prisco A. and Mauriello G. (2016). Probiotication of foods: a focus on microencapsulation tool. Trends in Food Science and Technology, 48, 27-39.
- De Prisco A., Maresca D., Ongeng D., Mauriello G. (2015). Microencapsulation by vibrating technology of the probiotic strain *Lactobacillus reuteri* DSM 17938 to enhance its survival in foods and in gastrointestinal environment. LWT - Food Science and Technology, 61, 452-462.
- Maresca D., De Prisco A., La Storia A., Cirillo T., Esposito F., Mauriello G. (2015). Microencapsulation of nisin in alginate beads by vibrating technology: preliminary investigation. LWT - Food Science and Technology, 66, 436-443.
- Vitaglione P., Troise A.D., De Prisco A., Mauriello G., Gokmen V. and Fogliano V. (2015) Use of microencapsulated ingredients in bakery products: technological and nutritional aspects. In Leonard M.C. Sagis Ed. Microcapsules and Microspheres for food Application. Elsevier, p-301-311.
- Mauriello, G., Maresca, D., Malmo C., De Prisco, A. (2013). Microencapsulation for functional foods: a focus on the Vibrating technology. New Food, 16 (5), 15-20.

Scientific Curriculum

Current occupation

PhD candidate
2017

March 2014- February

in Food and Agricultural Sciences at University of Naples, Federico II. Dept. of Agricultural Sciences, Portici (Na). Division of Microbiology.

Research topic: Study of the effects of microencapsulation on technological and functional feature of probiotic and starter cultures

Work experience

• **Research fellow**

January-October

Development of novel encapsulated ingredients, in particular antimicrobial substances (bacteriocins, essential oil).⁰¹³

In vitro and *in vivo* performance of encapsulated bioactives.

Activities within the program PON Research and Competitiveness 2007-2013. Project title "Encapsulation of active ingredients for improving the quality and food safety".

University of Naples, Federico II. Dept. of Agricultural Sciences, Portici (Na). Division of Microbiology.

• **Quality assurance**

January 2009-October
2012

Quality assurance and quality system management according to the standard UNI EN ISO 9001:2008 and IFS GSFS.

Production and packing in modified atmosphere of meat derivatives.

Jomi S.p.a. ,Via delle Industrie, 95, 80067-Bellizzi (Sa) and Via Roma, 50- Langhirano (Pr), Italy.

• **Quality control**

August- October 2006

Quality control and HACCP.

Production of tomato derivatives.

Europa Conserve ,Sant'Antonio Abate, 80059 (Na), Italy.

Education and training

23-26 February 2015

BRG 7th Training School on Microencapsulation

organized by Strasbourg University andmBioencapsulation

Research Group, held in Strasbourg University

- Overview and physiochemical methods for encapsulation;
- Practices in microcapsules characterization;
- Current research on living cells and functional ingredients encapsulation;
- Laboratory scale demonstrations;
- Physical methods and technologies for encapsulation;
- Pilote scale demonstrations;
- Scaling: from LAB to industrial production.

University of Strasbourg, France.

July 2012

Master degree in Food Science and Nutrition, with honours

with a thesis on the study of the encapsulation of oregano essential oil component (carvacrol) and its antimicrobial effect on food pathogens and spoilers. Title: "Encapsulation of natural antimicrobial substances: the case of carvacrol".

University of Naples, Federico II. Dept. of Agricultural Sciences, Portici (Na). Division of Microbiology.

July 2009

Bachelor degree in Food Science and Technology

with a thesis on the mechanisms related to programmed cell death in bacteria, with particular attention to two food pathogens (*E. coli* and *B. subtilis*). Title: "Apoptosis in bacterial cells".

University of Naples, Federico II. Dept. of Agricultural Sciences, Portici (Na). Division of Microbiology.

Publications

- De Prisco A., van Valenberg HJF, Fogliano V, Mauriello G. Behaviour of microencapsulated starter culture during yoghurt manufacturing. Submitted to Food and Bioprocess technology.
- Mauriello G., De Prisco A., Di Prisco G., La Stora A., Caprio E. Microbial characterization of bee pollen from the Vesuvius area collected by using three different traps. Submitted to PLOS ONE.
- De Prisco A. and Mauriello G. (2016). Probiotication of foods: a focus on microencapsulation tool. Trends in Food Science and Technology, 48, 27-39.
- De Prisco A., Maresca D., Ongeng D., Mauriello G. (2015). Microencapsulation by vibrating technology of the probiotic strain *Lactobacillus reuteri* DSM 17938 to enhance its survival in foods and in gastrointestinal environment. LWT - Food Science and Technology, 61, 452-

462.

- Maresca D., De Prisco A., La Stora A., Cirillo T., Esposito F., Mauriello G. (2015). Microencapsulation of nisin in alginate beads by vibrating technology: preliminary investigation. *LWT - Food Science and Technology*, 66, 436-443.
- Vitaglione P., Troise A.D., De Prisco A., Mauriello G., Gokmen V. and Fogliano V. (2015) Use of microencapsulated ingredients in bakery products: technological and nutritional aspects. In Leonard M.C. Sagis Ed. *Microcapsules and Microspheres for food Application*. Elsevier, p-301-311.
- Mauriello, G., Maresca, D., Malmo C., De Prisco, A. (2013). Microencapsulation for functional foods: a focus on the Vibrating technology. *New Food*, 16 (5), 15-20.

Conference participation and contributions

- De Prisco A. Mauriello G and Hein J.F. van Valenberg (2016). Behaviour of a microencapsulated starter culture during preparation, storage and simulated gastrointestinal transit of set yoghurt. *Food Micro 2016*, University College of Dublin. Abstract and poster n°779.
- De Prisco A. Mauriello G and Ongeng D. (2016). Survival of *Latobacillus reuteri* DSM 17938 microencapsulated by vibrating technology in three different microcapsules after heat treatments. *Food Micro 2016*, University College of Dublin. Abstract and poster n°780.
- Mauriello, G., Maresca, D., De Prisco, A., La Stora, A. (2013) Preservation of nisin by microencapsulation through vibrating technology. *International Congress Spoilers in Food*. Quimper France, 1-3 July. Poster pp. 136
- De Prisco, A., Maresca, D., Mauriello, G. (2013) Application of vibrating technology for microencapsulation of bacterial cells and nisin. "New frontiers in living cell encapsulation". Università degli studi di Perugia 8-10 Settembre 2013. Abstract pp. 24 and oral presentation.

Research and educational experience

Guest at Proteomics Laboratory at Veterinary Medicine,
University of Milan (Università degli Studi di Milano)

December
2016

Investigation of protein expression in free and encapsulated
Lactobacillus reuteri cells under simulated gastro-intestinal
transit.

PhD guest at Wageningen UR (Netherlands)- Group of Food
Quality and Design, Dairy division.

February – August 2016

Investigation of behaviour of microencapsulated probiotic

starter culture during yoghurt manufacturing, with special attention to acidification and growth kinetics, proteolysis and aroma-related volatiles production.

Guest at Gulu University, Faculty of Agriculture, Uganda within Gulu-Nap Project of University of Naples, Federico II.

August 2015

Set-up of molecular biology laboratory and training to future trainers on molecular biology techniques and experiments (e.g. bacterial DNA extraction, PCR of bacterial genes, electrophoresis).

Membership

- SIMTREA, Italian Society of Agri-Food and Environmental Microbiology.
- Bioencapsulation Research Group

January 2015

January 2015

