UNIVERSITY OF NAPLES "FEDERICO II"

DEPARTMENT OF AGRICULTURAL SCIENCES

AND

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FACULTY OF GRADUATE STUDIES

MASTER DEGREES IN

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AND

NUTRITION AND FOOD TECHNOLOGY

APPLICATION OF MICROENCAPSULATED BACTERIOCIN PRODUCER FOR AN ANTIMICROBIAL PACKAGING DEVELOPMENT

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Academic year 2020-2021

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Dedication

To my beloved parents, who raised me to believe that anything is possible, to my friend Rawa'a, who encouraged me to go on every adventure, especially this one, to my amazing brothers and sisters, who supported me until the completion of this research, and to my friends, colleagues, and relatives, I dedicate this work.

Acknowledgment

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And last but not least, all thanks and gratitude to the Universities of An-Najah National and Naples Federico II, for this effective joint program between them.

الاقرار

أنا الموقع أدناه، مقدّم الرسالة التي تحمل العنوان:

APPLICATION OF MICROENCAPSULATED BACTERIOCIN PRODUCER FOR AN ANTIMICROBIAL PACKAGING DEVELOPMENT

أقر بأن ما اشتملت عليه هذه الأطروحة إنما هو نتاج جهدي الخاص، باستثناء ما تمت الاشارة إليه حيثما ورد. وأن هذه الرسالة كاملة، أو اي جزء منها لم يقدم من قبل لنيل اي درجة او لقب علمي او بحثي لدى أي مؤسسة تعليمية أو بحثية أخرى.

Declaration

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

Student's Name: Mawadda Tyseer Jaber **اسم الطالب:**

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Date: 26/7/2021 **التاريخ:**

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APPLICATION OF MICROENCAPSULATED BACTERIOCIN PRODUCER FOR AN ANTIMICROBIAL PACKAGING DEVELOPMENT

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Abstract

This research aimed to study the viability and antimicrobial activity of bacteriocin-producing lactic acid bacteria (LAB) incorporated into edible film and the antimicrobial activity of the film against *L. innocua* in vitro system. The results of the work showed that the films were able to ensure high viability of the bacteriocin-producing strain *L. curvatus* 54M16 at 4°C during 28 days of storage. Moreover, it was investigated the effect of microencapsulation on the viability and antimicrobial activity of LAB to design edible film with microencapsulated strain.

Chapter One
Introduction

1.1 Food bio-preservation

Nowadays, the health benefits of "natural" and "conventional" foods, manufactured without added chemical preservatives, are becoming more attractive, as consumers are particularly aware of the health concerns related to food additives. Thus, in the past decades, food producers have faced conflicting challenges due to strict government requirements to provide food safety guarantees, as well as a growing consumer demand for high quality, natural foods (Settanni & Corsetti, 2008).

Foods technologies in which are involved microorganisms or their products to improve food safety and extends the shelf-life are referred to bio-preservation processes (Paul Ross *et al.*, 2002).

1.1.1 Food antimicrobial

Compounds that inhibit the growth of microorganisms or in some cases achieve complete cell death are called food antimicrobials (Davidson *et al.*, 2013). Most of them have been in use for 50 to 100 years or more; an example of one of the first groups of antimicrobials approved for use in foods is benzoic acid and its salts. Several conventional antimicrobials are approved in the United States for use in food products including benzoic acid in margarine and beverages, acetic acid in seasonings and baked goods, lactic acid in fermented foods and meat, sorbic acid in wine, nitrites

and nitrates in cured meats, and sulfates in potatoes and fruit products (Michael Davidson & Harrison, 2002). Only few antimicrobials are used solely to control the growth of specific bacterial pathogens although they are used to extend the shelf life and quality of foods and to inhibit spoilage microorganisms for many years. It is possible to derive a wide range of effective natural antimicrobial systems from plants, animals, and microorganisms. Examples of animal-derived antimicrobials are proteins such as lactoferrin, enzymes such as lysozymes, and small peptides such as histatins (Davidson *et al.*, 2013). However, due to the increasing number of consumers whose vegan or vegetarian diets restricts or prohibits the consumption of animal products; animal-derived natural antimicrobials are still viewed as undesirable. Therefore, it may be preferable to use natural antimicrobials derived from microorganisms (e.g., bacteriocins) or from plants (e.g., essential oils and phenols) to inhibit foodborne pathogens (O'Bryan *et al.*, 2018).

1.1.2 Bacteriocins

Bacteriocins exert antagonistic activity against a group of closely related and unrelated bacteria. They are small polypeptide molecules synthesized by the ribosome, and are one of the conservation strategies, to maintain intended food matrices (Johnson *et al.*, 2017).

There are generally accepted points to be common to many bacteriocins but they are by no means universal; one of them being that they are bactericidal proteins with a narrow inhibitory spectrum, especially for the same species

as the bacteriocin producer. The producing cell also dies because it is produced by lethal biosynthetic machinery, and it has been suggested that bacteriocins should be encoded by plasmid-borne genetic determinants, and bound to specific cellular receptors. The definition has broadened somewhat in recent years as "a protein-based or non-self-propagating protein complex, which exhibits growth inhibitory activity against a limited range of bacteria"(O'Bryan *et al.*, 2018).

1.1.2.1 Nomenclature and Classification of Bacteriocins

Bacteriocins were divided into four classes according to a classification procedure modified by Nes *et al.* (1996). Based on their chemical and physical properties, molecular weight, and an amino acid sequence, with the majority of those produced by bacteria associated with food belonging to Class I or II.

Class I bacteriocins (less than 5 kDa) consist of small membrane-active peptides that contain (unusual) lanthionine amino acids. The nisin A discovered by Rogers and Whittier (1928) is one of the oldest and most widely studied classes of bacteriocins. *Lactococcus lactis* (the lactic acid bacteria used in cheese making) produce nisin A (O'Bryan *et al.*, 2018). Carnocin U149, lacticin 481, and lactosin S are also examples of class I bacteriocins (Stoyanova *et al.*, 2012). Class II bacteriocins are small, thermo stable and thermally active non-lanthionine peptides. This class is divided into three subgroups IIa, IIb, and IIc. Bacteriocins IIa active against *Listeria* spp. include sakacin A, curvacin A, pediocin PA-1 (produced by

Pediococcus acidilactici, it is the most common type of bacteria known in IIa), leucocin A, sakacin P, and pediocin AcH (Perez *et al.*, 2014). Group IIb includes Lactococcin M, lactacin F, and Lactococcin G. Bacteriocins IIc include lactococcin B, which are thiol-activated peptides that require reduced cysteine residues for activity. Class III and Class IV bacteriocins are not well characterized. Class III bacteria (greater than 30 kDa) include helveticin V-1829, helveticin J, acidophilus A and lactacin A and B, which are temperature sensitive proteins (O'Bryan *et al.*, 2018). Class IV bacteriocins consist of complex bacteriocins, but information about this class is limited and contradictory (Franz *et al.*, 2007).

1.1.2.2 Mode of Action of Bacteriocins

Class, I bacteriocins form a complex with the bacterial cell wall lipid II precursor. The complexes assemble after cell wall biosynthesis is inhibited, then incorporate more peptides to form a physical pore through the bacterial membrane (Bierbaum & Sahl, 2009). Pore formation leads to cell death due to loss of membrane integrity and causes loss of intracellular ions and proton motive force (PMF). This leads to a loss of ability to transport protons and generate ATP due to disruption of chemical gradients across the membrane resulting from this loss. The activities of bifidocin B produced by *Bifidobacterium bifidum* NCFB have been reported to cause similar losses of K+ ions to sensitive cells (O'Bryan *et al.*, 2018).

1.2 The protective cultures

The nature of food it is a rich source of nutrients so it is a suitable habitat for attracting microbial colonization. Food spoilage occurs when the nutritional value, taste, and structure of the product are detrimentally affected due to the colonization of spoilage microorganisms that alter nutritional properties of the food. Fusarium, Aspergillus, Penicillium, and many strains of food spoilage fungi can produce mycotoxins that cause serious health problems once ingested. Their production occurs often when the fungus is in stress conditions to values of water activity, temperature, and amount of oxygen (Varsha & Nampoothiri, 2016).

Lactic Acid Bacteria (LAB) through its synergic effect inhibit the growth of a wide range of fungi. LAB produce a variety of antifungal compounds such as organic acids including hydroxyl fatty acids, and can be used as a protective culture without altering the organoleptic properties of food products (Florou-Paneri *et al.,* 2013). LAB occur naturally in various food sources and are classified as "generally recognized as safe" (GRAS, in the US) and with a Qualified Presumption of Safety (QPS, in the EU) (EFSA, 2016). LAB strains can be used as effective alternatives to food preservation as selected cultures or by using their metabolites (Varsha & Nampoothiri, 2016).

Protective cultures of LAB are natural inhabitant of fermented foods, used to improve the quality and shelf life of food products through the long-lived process of fermentation. Presence of different strains of LAB and

their function in fermented foods such as beer- takju, khanomjeen; fermented vegetable- kimchi, rice- wine, noodle- idli, tapuy; acid leavened bread, nham, puto, narezushi, burong; fermented fish and meat- sikhae, noodle- idli are well described (Rhee *et al.*, 2011). LAB have been effectively used to control the growth of *L. monocytogenes*, *S. Typhimurium* in delicious golden apples and iceberg lettuce, and the growth of *salmonella enteritidis* and *Listeria monocytogenes* in chicken meat (Varsha & Nampoothiri, 2016).

1.3 The microencapsulation

1.3.1 Generalities on microencapsulation technologies

From the principle of cell immobilization, evolved the technique of microencapsulation. In a broader sense, this technique can retain sensitive compounds onto/ within functional matrices as shown in the figure 1.1 the microencapsulation step involves the complete encapsulation of the selected base materials in a gaseous, liquid, or solid phase within an impermeable membrane, or defined natural or synthetic porous membrane by using various techniques (De Prisco & Mauriello, 2017).

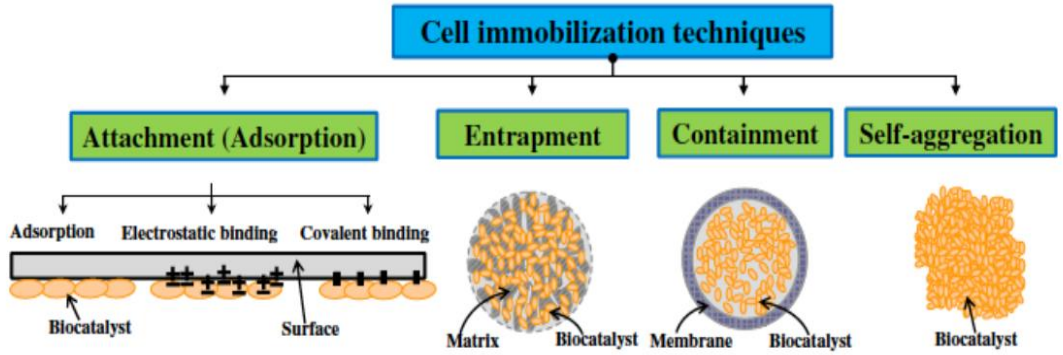

Fig. 1.1 Types of cell immobilization techniques. Abdel-Rahaman *et al.*, 2013.

Microcapsules can be classified according to their morphology into five different main types as shown in the figure 1.2. The capsule size varies from Nano (<1 µm), micro (1-1000 µm), or macro (>1000 µm) (Solanki *et al.*, 2013). Microencapsulation is usually defined as an encapsulation technique applied to microbial cells, which are typically 1-4 µm in size (Gawkowski & Chikindas, 2013).

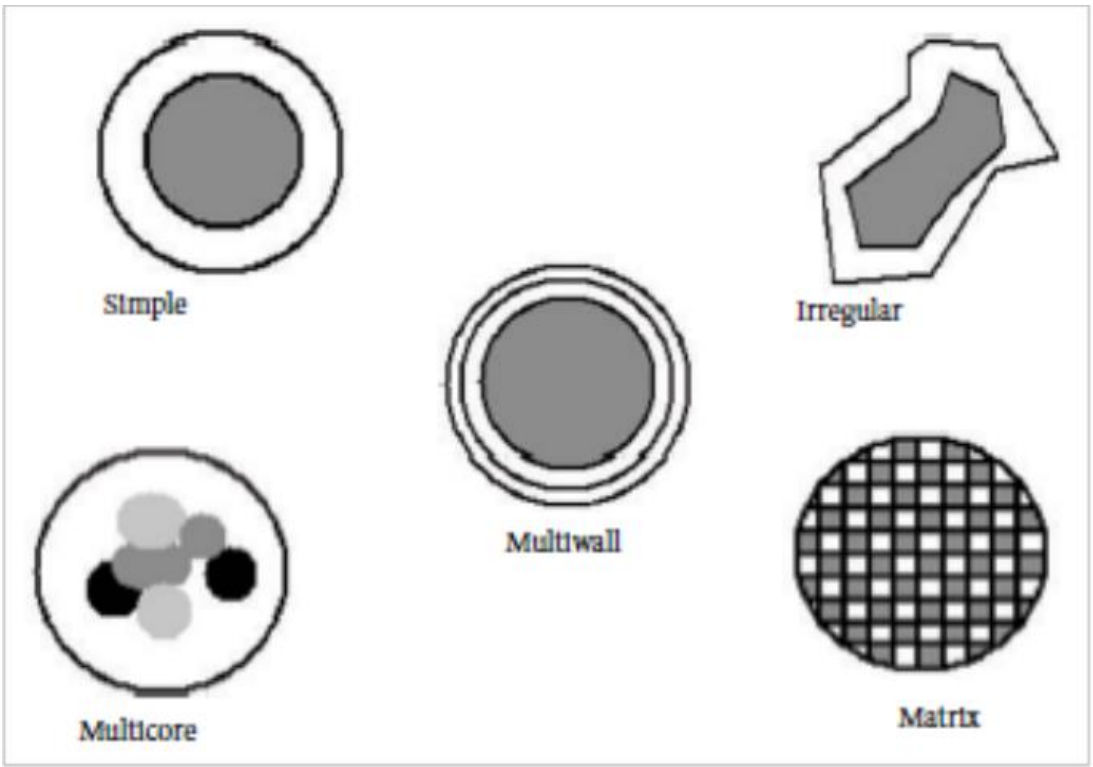

Fig. 1.2 Different types of microcapsules used in food industry. Solanki *et al.*, 2013.

In the "matrix" type of microencapsulation, between the encapsulating component and the encapsulating agent there is no physical separation because the encapsulating component is present throughout the entire microcapsule region, homogeneously dispersed to create a network with the encapsulating agent (De Prisco & Mauriello, 2017). The encapsulated components in the case of a "multi-core" system form two or more groups of assemblies, as the case of emulsification techniques (Whelehan & Marison, 2011). In the “reservoir” or “core-shell” microcapsule, the core diameter and membrane thickness can vary, as cells or other sensitive compounds are kept in the core of the capsules completely surrounded by a continuous enclosed membrane (Whelehan & Marison, 2011). As for the “irregular” shape, the capsules do not have a well-defined spherical morphology.

“Multi-walled” microcapsules are particles resulting from the possibility of applying another layer of coating agent to all the previously mentioned types of microcapsules which can create bonds with the capsule material due to chemical or physical reactions. Through many different microencapsulation processes and a wide range of wall materials including centrifugal extrusion, air-suspension coating, spray-drying, extrusion, spray-cooling, coacervation, membrane emulsification, interfacial polymerization, spray-chilling, emulsion or microchannel, co-crystallization, freeze-drying, and liposome entrapment, different types of microcapsules are produced (de Vos *et al.*, 2010).

1.3.2 Microencapsulation of microbial cells

To date, fermented and non-fermented dairy products remain among the probiotic foods available in the market most consumed by the population. Species of *Bifidobacterium* and *Lactobacillus* are the most common probiotic bacteria added to foods. Other genera (*Escherichia*, *Enterococcus*, and *Bacillus*) include strains that are recognized as probiotics but their main use is in the production of nutritional supplements.

Various factors can interfere with the basic requirements for probiotics to achieve a lively gut and metabolic activity, affecting the vigor and behavior of microorganisms in different food environments, so they must be considered, subcellular injury by heat, type of culture chosen, osmotic stress, or stage of the growth and as for external factors, storage time, pH value, food processing conditions, and oxygen level (Soukoulis *et al.*, 2013). The bile and pH are major stress agents that cause the loss of viability of the probiotics during gastrointestinal transit. Therefore, probiotics must be able to resist to these stress agents to perform their biological role. To improve the durability of technological and gastrointestinal probiotics various strategies have been applied to obtain "ultra-fit" bacteria; genetic manipulation, careful selection of strains with the highest local technological positions, strain adaptation to a sub-lethal dose of a specific chemical or physical stress, use of cell-protectants (Gueimonde & Sánchez, 2012), in addition to a technological approach that

deals with the inclusion of probiotics in microcapsules (Rokka & Rantamäki, 2010), or edible films (Soukoulis *et al.*, 2014). The main purpose or at least the most researched aspect to date of bacterial cell microencapsulation as described by some authors; is the protection of cells under gastrointestinal conditions (Sohail *et al.*, 2011).

However, another challenge of microencapsulation, such as in bakery products, is the protection of probiotic cells in foods that are not normally considered a vehicle for probiotics (Malmo *et al.*, 2013). Several advantages, apart from the effects mentioned above, can be offered by microencapsulation in masking the odor and taste resulting from the production of various metabolic compounds produced during fermentation in foods that are not required (such as acetic acid) as well as promoting probiotic cultures. Improving the survival of probiotics after its inclusion in the powder formulation is an additional important aspect of the encapsulation efficacy as there is a detrimental effect of reduced oxygen exposure and water activity (Weinbreck *et al.*, 2010).

1.4 Antimicrobial packaging

One of the many applications of active packaging is antimicrobial packaging, which is a packaging system capable of preventing the contamination or killing pathogenic and spoilage microorganisms. By using antimicrobial polymers and/or adding antimicrobial agents in the packaging system an antimicrobial function that meets the requirements of conventional packaging can be achieved. Antimicrobial packaging should

maintain food quality and safety and extend shelf life by reducing the growth rate of microorganisms and prolonging the lag phase (La Storia & Mauriello, 2008).

1.4.1 Developing the antimicrobial packaging systems

Most food packaging systems represent either a package/headspace/food system or a package/food system as shown in the **figure 2.3**.

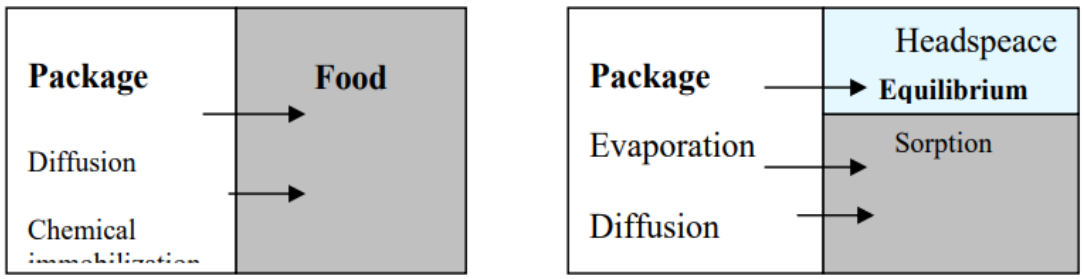

Fig. 1.3 Food packaging systems and relative behavior of active substances (La Storia & Mauriello, 2008).

Package/headspace/food systems represented by foods packaged in cartons, flexible packages, and cups, equilibrated or balanced evaporation should be considered as part of the main relay mechanisms for estimating the interfacial distribution of the material in this packaging system. The other system, which is the packaging/food system, is the contact of the packing material with the solid food product, or liquid or low-viscosity food without a vacuum. One of the most important phenomena of migration involved in this system is diffusion between food and packaging materials and partitioning at the interface. Antimicrobial agents can initially be incorporated into the packaging materials and then migrate to the food

through partitioning and diffusion. In these systems, a volatile active substance can be used as it can travel through the air gaps and headspace between the food and the package (La Storia & Mauriello, 2008).

Antimicrobial packaging can take many forms, for example; the use of antimicrobial polymers, incorporation of antimicrobial agents (volatile and non-volatile) directly into polymers, the addition of dressings/sachets containing antimicrobial agents (volatile) to packages, antimicrobial stabilization of polymers by covalent bonds or ions, or on polymer surfaces Coating and antimicrobial absorbent (La Storia & Mauriello, 2008).

1.4.2 Antimicrobial compounds incorporated in the edible films

The additives used to inhibit the growth of pathogenic micro-organisms and to control biological degradation, are known as antimicrobial compounds. Natural extracts (Essential oils, Plant and/or spice extracts, Enzyme, Bacteriocins), chemical agents (Organic acids and their salts like; citric acid, sorbic acid or benzoic acid, acetic acid, lactic acid, and their salts), or probiotics (*Bifidobacterium* and *Lactobacillus*, although *Saccharomyces boulardii* and *Streptococcus thermophilus*) can be incorporated into edible films as antimicrobial compounds. The selection of antimicrobial compounds for food packaging materials should be based on the release mechanisms of active substances in foods, toxicity and regulatory issues, the nature of the active agents and their inhibition mechanisms, the packaging manufacturing process, and its effect on the efficiency of the active agents, the sensory properties of the active

compounds and the chemical and physical properties of foods, the Physiology of target microorganisms and micro flora of foods, and storage conditions (Rawdkuen, 2019).

Chapter Two
Materials and Methods

2.1 Microorganisms and cultivation

2.1.1 Bacterial strains and growth conditions

Lactobacillus curvatus 54M16 was isolated from traditional fermented sausages and produces the bacteriocins sak X, sak Tα, sak Tβ and sak P (Casaburi *et al.,* 2016; Giello *et al.,* 2018). The strain was stored at - 20 °C in MRS broth (Oxoid) supplemented with 25% (v/v) sterile glycerol. Before use, the strain was sub-cultured twice in a modified MRS broth (m-MRS) at 30 °C. The composition of the m-MRS broth was (g/L of distilled water): 8.0 Lab-Lemco, 10 Peptone, 4.0 Yeast Extract, 20 Dextrose, Tween 80 (1 ml/L). A 10 ml aliquot of reactivated culture was transferred into 1000 ml of MRS broth and incubated for 24 h at 30 °C.

Listeria innocua 1770, used as bacteriocin indicator, was grown in Trypticase Soy Broth supplemented with 0.5% yeast extract (TSBY, Oxoid) at 30 °C.

2.1.2 Film preparation

40 ml of culture broth of *Lb. curvatus* 54M16 was centrifuged at 6500 rpm for 15 min. The pellet was re-suspended in 4 ml of MRS and 1 ml of glycerol and frozen at - 20 °C. At the time of use, the broth culture was thawed, centrifuged at 6500 rpm for 15 min, and the pellet re-suspended in 4 ml of m-MRS for including into the film formulation (**Table 2.1**). Two

different films were prepared: a) control film prepared without *Lb. curvatus* 54M16 (FC); b) bioactive film prepared adding *Lb. curvatus* 54M16 suspended in m-MRS broth (FL). Culture of *Lb. curvatus* 54M16 was added to film forming solutions at 4% (v/v) to obtain a final concentration of 8.20 Log CFU/mL for FL. Sodium caseinate, glycerol, guar gum, beeswax, surfactants and m-MRS broth were dissolved in deionized water under continuous stirring at 50 °C for 30 min. Then, the solution was cooled down to 30 °C and enriched with *Lb. curvatus* 54M16 at 4% (v/v) under continuous stirring for 15 min (FL).

Films were obtained by casting five ml of each mixture were poured into Petri dishes (surface of 56.7 cm^2) and allowed to dry at 30 °C and 50% relative humidity (RH) for 24 h in circulating air system chamber. Dried films were peeled from the Petri dishes and stored at 4 °C and 50% RH prior to testing.

Table 2.1: Composition of the films.

Composition	**Films** [a]	
	FC	**FL**
Sodium caseinate (% w / v)	8	8
Glycerol (% w / v)	0,8	0,8
Guar gum (% w / v)	0,2	0,2
Beeswax (% w / v)	2	2
Surfactants (% v / v)	0,25	0,25
m-MRS (% v / v)	15	15
Broth culture *Lb. curvatus* 54M16 (% v / v)	0	4
Deionized water (% v / v)	73,75	69,75
a: FC, control film prepared without *Lb. curvatus* 54M16; FL, film prepared with the addition of *Lb. curvatus* 54M16.		

2.1.3 Viability of *L. curvatus* 54M16 during storage of the film

Viable counts of *L. curvatus* 54M16 were determined in the broth culture before adding in the film solution and immediately after got the film solution, while the viability of the microorganisms was determined in the films after 1, 7, 15 and 28 days of storage at 4 °C. The broth culture and the film solution were diluted 1:10 in quarter-strength Ringers solution (Oxoid) and aliquots of serial decimal dilutions were poured in duplicate plates of MRS agar. The plates were incubated at 30 °C for 48 h. The results were expressed as log CFU/ml. The films (FL and FC) at each sampling time were placed in a Stomacher bag with 56.7 ml of Ringers solution (Oxoid). The bag containing each film was homogenized by stomacher for 2 min. Sequential decimal dilutions were made in Ringer's solution. Each dilution was plated on MRS agar and incubated at 30 °C for 48 h. The results were expressed as means of log CFU/cm^2.

2.2 Assessment of the antimicrobial activity

Antimicrobial activity of the films during storage at 4 °C was detected against the strain of *L. innocua* 1770. At each sampling time, film pieces (ca 2x2 cm^2) were aseptically cut from each film and placed on TSA soft agar (0.75% agar) plates inoculated at 1% with an overnight culture of the indicator strain. Plates were incubated at 30 °C, 10 °C, 20°C and 4 °C for 24 and 72 h, 4 days and 7 days, respectively. After incubation the inhibition zones of the indicator organism around the films were determined and expressed in cm^2. Each value was the mean of two experiments with three

replicates each. The antagonistic activity of the active films was also evaluated by determining *L. innocua* counts, as described by Sanchez-Gonzalez, Quintero Saavedra, and Chiralt (2014), with some modifications. Overnight culture of *L. innocua* 1770 was inoculated (about 10^5 CFU/cm^2) on the surface of solidified TSA plates, which were then covered with active (FL) and non-active (FC) films with the same size as the Petri dishes. The counts of *L. curvatus* 54M16 and *L. innocua* 1770 were determined after 0, 1, 7, 15 and 28 days of storage at 10°C. At each sampling time the agar covered with the films was withdrawn aseptically from the Petri dishes and transferred in a Stomacher bag with 100 ml of quarter strength Ringer's solution (Oxoid). The content of the bag was homogenized by stomacher for 5 min. Sequential decimal dilutions were made in Ringer's solution. Each dilution was plated on MRS agar incubated at 30 °C for 48 h and on ALOA (Biolife, Milano, Italy) incubated at 30 °C for 48 h. The results were expressed as means of log CFU/$cm^{2.}$

2.3 Microencapsulation of the bacteriocin producer

2.3.1 Bacterial cells microencapsulation in alginic acid solution

Using the Encapsulator B-395 Pro (BUCHI, 120 Switzerland) microencapsulation of strain 54M16 was carried out as shown in the figure 2.1. Overnight culture (final volume 2 L) was centrifuged for 15 min at 6500 g. Sterile water was used to wash the cell pellets (3.20 g), then resuspended in 30 ml of 17 g/L alginic acid sodium salt after harvesting by centrifugation. Through the nozzle (80 µm) the alginate-cell suspension

was forced into the pulse chamber and extruded after being loaded into the syringe. The conditions for the microencapsulation used were: electrode voltage of 1200 V, vibration frequency 1800 Hz, a flow rate of 3.89 ml/min. By hardening the droplets in 1 L of a sterile 0.5 mol/L CaCl2 solution, microcapsules containing cells were obtained with constant stirring at 100 rpm. Microcapsules (MAlg) after sedimentation and gentle shaking of the CaCl2 solution are stored in sterile water at 4 and 30 °C for further experiments (De Prisco & Mauriello, 2017).

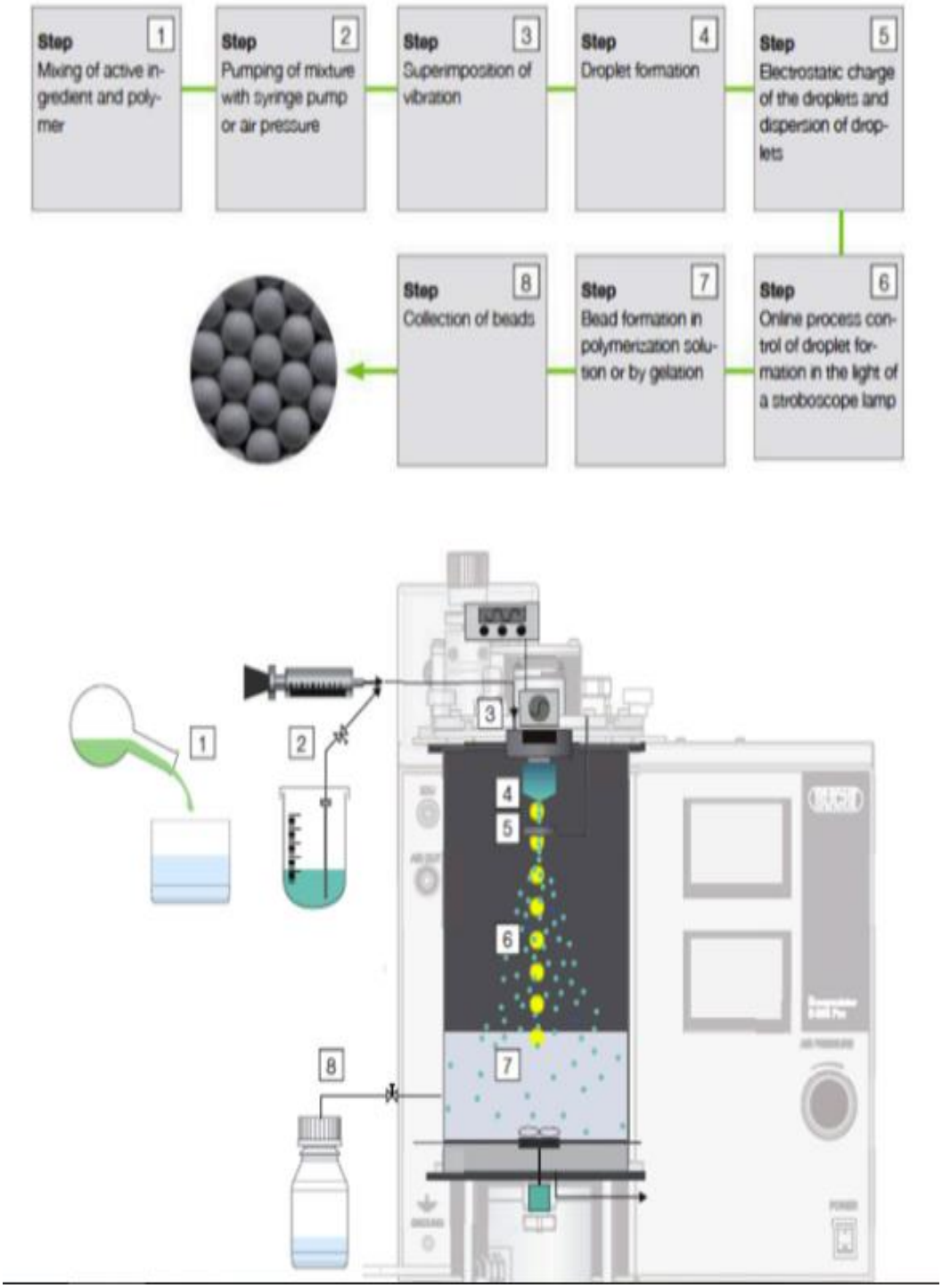

Fig. 2.1 Functional principle of vibrating technology using BUCHI Encapsulator B-395 Pro (BUCHI; Switzerland; De Prisco *et al.*, 2015)

2.3.2 Bacterial cells microencapsulation in alginic acid and mMRS solution

In this experiment, in 30 ml of 17 g/L alginic acid sodium salt, the cell pellet was suspended. The alginic acid sodium salt was enriched with some components of MRS broth (Dextrose 20 g/L, Peptone 10 g/L, Tween 80 1 ml/L, Lab Lembo 8 g/L, yeast extract 4 g/L). As previously described, microencapsulation was carried out. Microcapsules (MAlg + MRS) were stored in sterile water at 30 and 4 °C for further experiments (De Prisco & Mauriello, 2017).

2.3.3 Morphology of microcapsules

Using a Zeiss light microscope (200X magnification and calibrated μm) the surface morphology and size of the microcapsules were analyzed (De Prisco & Mauriello, 2017).

2.3.4 Efficiency of microencapsulation process

By dividing the “viable number of inactivated microcapsules” by the “cell load of the alginate-cell suspension”, the efficiency of the microcapsules was calculated. Using an equal volume of 0.1 mol/L phosphate buffer solution the microcapsules were inactivated at pH 7.0. And by microscopic observation, the disruption of the microcapsules was confirmed. Also on the integer microcapsules, the Cell loading was determined. On MRS agar for 24 h at 30 °C viable cell counting was performed (De Prisco & Mauriello, 2017).

2.3.5 Antimicrobial activity of MAlg and MAlg + MRS in resting cell condition

Qualitative determination (absence/presence of an inhibition halo) of bacteriocin MAlg activity was evaluated at 4° and 30°C at T0 (immediately after microencapsulation), as well as after 24, 48, 72, and 144 h of storage. Qualitative determination of the bacteriocin activity of MAlg + MRS was evaluated at 4 and 30 °C after 0, 24, and 96 h of storage. In particular, wells (6 mm diameter) bored into TSA agar plates containing approximately 10^6 CFU/ml of *Listeria innocua* 1770 indicator strain were worked, then through the pipetted was transferred 50 μl of sterile water in contact with the, disrupted microcapsules (DM), MAlg and MAlg + MRS, and integer (M). Then the plates were incubated at 30°C overnight (De Prisco & Mauriello, 2017).

2.3.6 Antimicrobial activity of MAlg in modified MRS (mMRS)

1 g of microencapsulated strain (1.7 x 10^9 CFU/ml) and 1 ml of 54M16 overnight culture (2.6 x 10^8 CFU/ml) were inoculated in 10 ml of mMRS (Tween 80 1 ml/L, Peptone 10 g/L, yeast extract 4 g/L, Lab Lembo 8 g/L) and incubated at 30 °C. Previously centrifuged and filtrated microencapsulated strain (mMRSMic), mMRS inoculated with free (mMRSFree) and integer microcapsules (M), at different time points 0, 24, 48, 72 and 144 h, 50 μl were pipetted into wells (6 mm diameter) bored into TSA agar plates containing about 10^6 CFU/ml of the indicator strain *Listeria innocua* 1770. The plates were incubated at 30 °C overnight. As

for the qualitative determination of bacteriocin activity, it was expressed by the presence or absence of the inhibition halo (De Prisco & Mauriello, 2017).

Chapter Three
Results

3.1 Viability of *Lb. curvatus* 54M16 during storage of the film at 4°C

The results are summarized in the table 3.1. It was noted that the viability of *Lb. curvatus* 54M16 inside the film (FL) after 28 days of storage at a temperature of 4°C was fairly stable and the decrease was very slight compared to the first day (from 7.25 to 7.03 CFU/cm^2).

Table 3.1: Viability of *Lb. curvatus* 54M16 during storage of the film at 4°C.

Films [a]	**Log CFU/cm^2 ± sd at days** [b]			
	1	**7**	**15**	**28**
FC	< 1	< 1	< 1	< 1
FL	7.25±0.17	7.31±0.18	7.29±0.19	7.03±0.50

a: FC, control film without *Lb. curvatus* 54M16; FL, film with *Lb. curvatus* 54M16.
b: 1, films immediately after the casting at 30°C for 24 h; 7, 15 and 28: films after 7, 15 and 28 days of storing at 4 °C.

3.2 Antimicrobial activity against *L. innocua* during storage of the film

Antimicrobial activity of the films during storage at 4 °C for 28 days was determined on plates of *L. innocua* 1770 incubated at 30°C, 20°C, 10°C and 4°C by measuring the inhibition halo.

As shown in Table 3.2, no significant decrease in activity was detected during the storage period for plates with FL films incubated at 30°C, 20°C, 10°C and 4°C. Moreover, control films (FC) prepared without *Lb. curvatus* 54M16 did not show antimicrobial activity against the indicator strain. The highest inhibition halos were observed at 4°C of incubation with 14.85 cm^2

and 14.42 cm^2 at 7^{th} and 28^{th} day, respectively. This assay highlight that the inhibition areas were significantly wider when the antagonism was detected at lower temperatures 4°C and 10°C (Table 3.2, Fig. 3.1). This may be due to a better diffusion of the bacteriocins from the film and/or to different growth dynamics of *L. innocua* 1770 in the presence of bacteriocins at temperatures of 4°C, 10°C, 20°C and 30°C (La Storia *et al.*, 2020).

Table 3.2: Antimicrobial activity against *L. innocua* during storage of the film.

Time (day)[c]	**Inhibition zones (cm^2)[a] ± sd at temperatures [b]**			
	30°C	**20°C**	**10°C**	**4°C**
0	8.12±0.4	9.75±0.21	14.07±0.53	14.13±2.65
7	8.12±0.4	9.45±0.64	12.95±4.21	14.85±1.63
15	8.12±0.4	8.75±0.33	13.10±0.96	14.25±1.01
28	8.12±0.4	9.07±0.82	12.95±1.24	14.42±1.72

a: inhibition halo including the area of the film (4 cm^2)
b: plates incubated at 30°C, 20°C, 10°C and 4°C for 24h, 72h, 4 days and 7 days, respectively.
c: 0, films immediately after the casting at 30°C for 24 h; 7, 15 and 28: films after 7, 15 e 28 days of storing at each temperature.

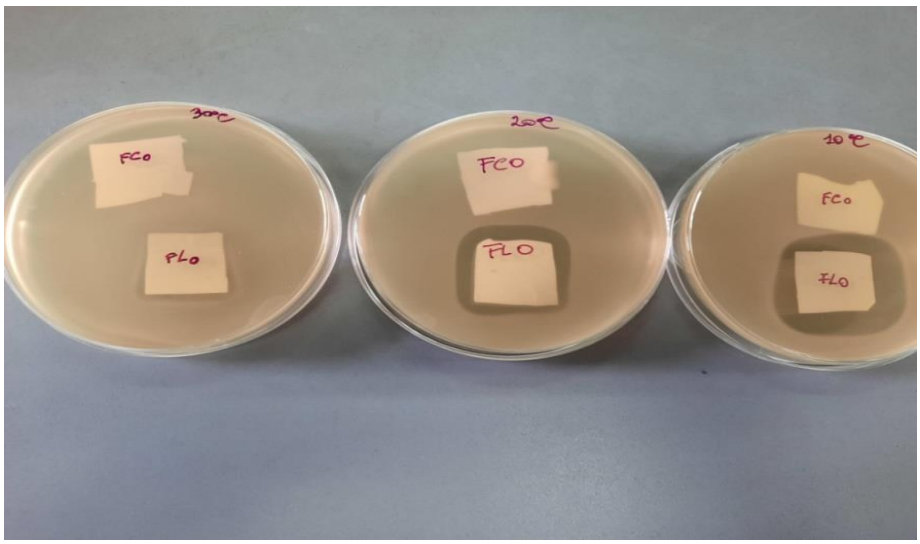

Fig. 3.1: Examples of inhibition zones of FL against *L. innocua* 1770 at different temperature of incubation. From the left: plate incubated at 30°C with FC up and FL down; plate incubated at 20°C with FC up and FL down; plate incubated at 10°C with FC up and FL down.

3.3 Antimicrobial activity against *L. innocua* on TSA during storage of the film at 10°C

The antimicrobial activity was also evaluated against *L. innocua* C6 inoculated (about 10^5 CFU/cm^2) on the surface of TSA plates that were then covered with FL and FC films and stored at 10°C for 28 days. The results of *L. innocua* counts (Table 3.3) showed that the FL film determined a significant decrease of the indicator strain compared to the control film (FC). The decrease of *L. innocua* was more than 2 log cycles after only 24 h, reaching values below the detection limits after 15 days until the end of the storage period. The results confirmed the bactericidal action of the bacteriocin produced by *Lb. curvatus* 54M16 (Giello *et al.*, 2018; La Storia *et al.*, 2020). On the contrary, the film without *Lb. curvatus* 54M16 (FC) did not cause inhibition of *L. innocua* that increased from 4.44 to 9.20 Log CFU/cm^2 after 28 days of storage. Moreover, *Lb. curvatus* count remained somewhat stable after the contact FL with the surface of the medium during the 28 days of storage at 10°C (Table 3.3). However, the bacteriocin-producing strain assured the protective effect of the film throughout the storage period.

Table 3.3: Antimicrobial activity against *L. innocua* on TSA during storage of the film at 10°C.

Time (day)[a]	Log CFU/cm^2 ±sd of *L. innocua* 1770		Log CFU/cm^2 ±sd of *Lb. curvatus* 54M16
	FL [b]	FC	FL
0	4.37±0.01	4.44±0.03	7.25±0.22
1	2.17±0.18	5.71±0.06	7.23±0.01
7	0.24±0.33	9.95±0.15	7.75±0.01
15	0	9.52±0.02	6.86±0.20
28	0	9.20±0.22	6.64±0.40

a: 0, samples after 15 min of contact of film on agarized medium; 1, samples after 24 h of storing at 10°C; 7, 15 and 28 samples after 7, 15 and 28 days of storing at 10 °C.
b: FC, TSA plates spreaded with *L. innocua* 1770 and covered by control film without *Lb. curvatus* 54M16; FL, TSA plates spreaded with *L. innocua* 1770 and covered by film with *Lb. curvatus* 54M16.

3.4 Microcapsules morphology

Both MAlg and MAlg + MRS microcapsules exhibited a regular spherical shape, and 54M16 cells were homogeneously distributed in the alginate matrix. The microcapsules showed an average diameter of about 150 µm (Fig. 3.1).

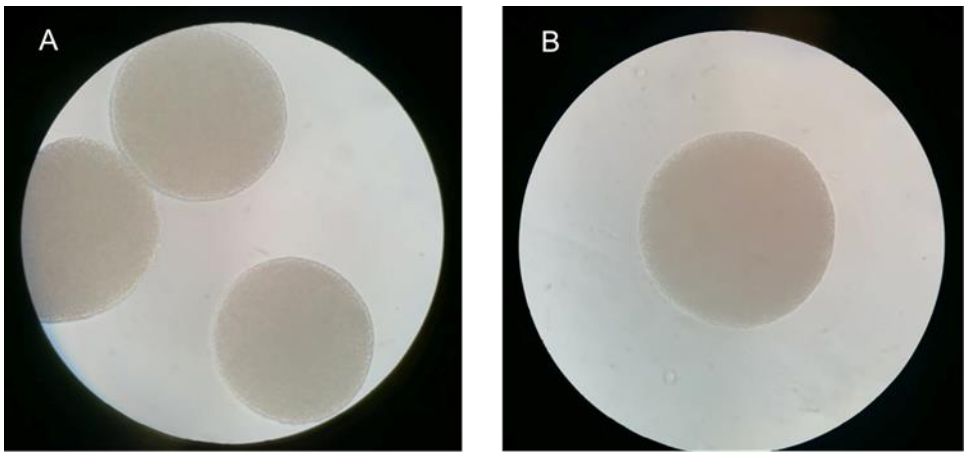

Fig. 3.2: Light microscopy images of MAlg (A) and MAlg + MRS (B).

3.5 Microencapsulation efficiency

In Table 3.4, the results of the bacterial load before and after microencapsulation are shown, to assess the efficiency of microencapsulation experiments. The viable counts of disrupted microcapsules indicated a reduction of 0.20 Log cycles only in the cell load with respect to the alginate-cell suspension, indicating efficiency of about 98% for microencapsulation.

Table 3.4: Viable counts (Log CFU/ml) of 54M16 strains before and after microencapsulation process.

Samples	Log CFU/ml
Alginate-cell suspension	10.50
Integer microcapsules	9.32
Disrupted microcapsules	10.30

3.6 Qualitative estimation of bacteriocin activity of MAlg and MAlg + MRS in resting cell condition

In Table 3.5, the results of the antimicrobial activity of MAlg are summarized. No inhibition halo was shown against the indicator strain in (S) contact water, stored at 4 °C after all incubation times (0, 24, 48, 72, and 144 h). On the contrary, in the disrupted MAlg (DM) an inhibition halo of about 1 cm, and about 0.6 cm for integer MAlg (M) was observed at all-time points.

At 30 °C, and only after 48, 72 hours of storage, water in contact with MAlg (S) showed an inhibition halo of about 0.8 cm. As for disrupted and

integer MAlg, an inhibition halo of about 0.6 and 0.4, respectively, appears after all time points.

In Table 3.6, the results regarding the antimicrobial activity of MAlg + MRS are summarized. No inhibition halo appeared after 24 hours of incubation in both water samples (S) in contact with microcapsules at 4°C and 30°C. A small halo was observed for disrupted and integer MAlg + MRS of only 0.4cm. An inhibition halo of approximately 0.5, 0.6, and 0.4 cm was also observed, at 4 °C and 30 °C after 96 h, in water in contact with MAlg + MRS, disrupted and integer MAlg + MRS, respectively.

Table 3.5: Qualitative estimation of bacteriocin activity (presence (cm) or absence (-) of inhibition halo) of MAlg after different incubation times (0, 24, 48, 72 h) in different store conditions (4 °C and 30 °C).

Samples (MAlg)	Store conditions (°C)/incubation times (h)									
	4 °C					30 °C				
	0	24	48	72	144	0	24	48	72	144
S	-	-	-	-	-	-	-	0.8	0.8	-
M	0.6	0.6	0.6	0.6	0.6	0.4	0.4	0.4	0.4	0.4
DM	1	1	1	1	1	0.6	0.6	0.6	0.6	0.6

Table 3.6: Qualitative estimation of bacteriocin activity (presence (cm) or absence (-) of inhibition halo) of MAlg + MRS after different incubation times (0, 24, 96 h) in different store conditions (4 °C and 30 °C).

Samples (MAlg + MRS)	Store conditions (°C)/incubation times (h)					
	4°C			30°C		
	0	24	96	0	24	96
S	-	-	0.5	-	-	0.5
M	0.4	0.4	0.4	0.4	0.4	0.4
DM	0.4	0.4	0.6	0.4	0.4	0.6

3.7 Qualitative estimation of bacteriocin activity of microencapsulated 54 M16 strain in mMRS

In **Table 4.7** all results are summarized, no inhibition halo appeared at 30 °C after 24 h of incubation in mMRS in both mMRSMic and mMRSFree samples. For M, inhibition halo of about 0.5 cm was recorded. An inhibition halo of about 0.4 and 1.2cm was observed for mMRSMic and mMRSFree, after 48 and 72 hours, respectively. After 144 hours, we did not record the inhibition halos for all samples.

Table 3.7: Qualitative estimation of bacteriocin activity (presence (cm) or absence (-) of inhibition halo) of microencapsulated 54M16 strain in mMRS after different incubation times (0, 24, 48, 72 h) at 30 °C.

Samples	Incubation times (h) at 30 °C				
	0	**24**	**48**	**72**	**144**
mMRSFree	-	-	1.2	1.2	-
mMRSMic	-	-	0.5	0.5	-
M	0.5	0.5	0.4	0.4	-

3.8 Qualitative estimation of bacteriocin release from MAlg + MRS in resting cell condition

Bacteriocin release from MAlg + MRS microcapsules (microencapsulation of 54M16 cell pellet and some ingredients of MRS broth medium: Peptone 10 g/L, Lab Lembo 8 g/L, yeast extract 4 g/L, Tween 80 1 ml/L, Dextrose 20 g/L) was evaluated after 0, 24, 72, 96 and 120 h (hours) of storage in water at different pH values (2.0, 4.0 and 6.0) adjusted with HCl 0.1 mol/L at 30 °C. After each time, different samples of water in contact with the microcapsules were retrieved and frozen for 24 h, at −18 °C. The samples were then subjected to a freeze-drying process (Thermo Scientific Heto

Power Dry PL6000). For further experiments, the lyophilized samples were resuspended in 500 µl of sterile water and stored at 4 °C. For the qualitative determination of bacteriocin activity by the presence or absence of the inhibition halo, 50 µl of each sample was pipetted at different time points: 0, 24, 72, 96, and 120 h; pH 2.0, 4.0, and 6.0, into wells (diameter 6 mm) bored into TSA agar plates containing approximately 10^6 CFU/ml of the indicator strain *Listeria innocua* 1770. The plates were incubated overnight at 37 °C. In Figure 3.2 the results are shown. Antimicrobial activity was observed after 24 h only for samples at pH 4.0 and 6.0 until the end of incubation, while samples at pH 2.0 did not show antimicrobial activity at each time of storage.

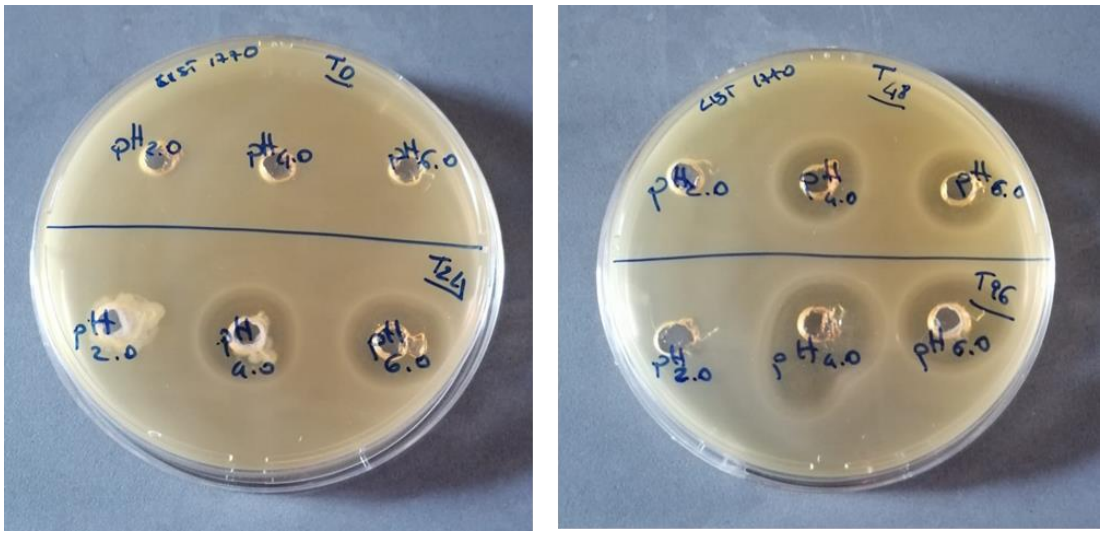

Fig. 3.3: Qualitative estimation of bacteriocin release from MAlg + MRS in resting cell condition.

Chapter Four
Discussion and Conclusion

4.1 Discussion

To ensure the effectiveness of the active films, ensuring the functionality and viability of the microorganisms incorporated into the biopolymer matrix are the main conditions.

The viability of the *Lb.curvatus* 54M16 strain producing bacteriocins contained in the film (FL) was determined while the films were stored at 4 °C for 28 days as shown in the table 3.1. It was observed that the number of bacteria was stable at a level ranging from 7.25 to 7.03 log CFU/cm2 during the storage period from the first day to 28 days (a very slight decrease of 0.22 units), which means that it is good that they remained alive and did not die, which indicates the formation of the film affects the vitality of the microorganism due to the presence of nutrients (m-MRS). Previous studies show that similar films based on glycerol and others, ensure the survival of different strains of lactic bacteria during storage and use as coatings for different foods (Shoaib *et al.*, 2016; Odila Pereira *et al.*, 2019; Argueta *et al.*, 2016).

The results of this study showed that to ensure the high viability of the bacteria-producing strain *L. curvatus* 54M16, the optimal composition of the edible films was: 8% Sodium caseinate, 0.8% glycerol, 0.2% Guar gum, 2% Beeswax, 0.25% Surfactants, 15% m-MRS, 4% Broth culture *Lb.*

curvatus 54M16, 69.75% Deionized water (Table 2.1). Therefore, the film can be considered a good vector for viable cells.

Based on the results listed in the table 3.2, it was observed that the area of the halo produced by the antimicrobial activity against *L. innocua* 1776 during storage at different temperatures for different periods inside the film; It increases with decreasing storage temperature, where the largest inhibiting area of *L. innocua* was at 4 °C, which is the temperature of the refrigerator (14.42 cm^2 after 28 storage days). To detect the antimicrobial activity of the membranes in all experiments *L. innocua* C6 strain was used as a live indicator. When comparing the *L. innocua* C6 with *L. monocytogenes* ATCC 7644 showed a similar growth rate but lower sensitivity to bacteriocins produced by *L. curvatus* 54M16 (Casaburi *et al.*, 2016).

The results showed that the temperature at which the antagonism was detected and the presence of nutrients were critical for the antimicrobial activity of the films. As shown in the table 3.2, a decrease in the antimicrobial activity of the film was observed after 28 days of storage at 4°C compared to the activity after 7 days of storage. The decrease in activity is attributed to the possible death of bacteria due to the decrease in nutrients during the film storage (Concha-Meyer *et al.*, 2011). Previous literature reviews have confirmed these and other findings; the choice of the nature of the film matrix and microorganisms are conditions for the

bacterial viability and antimicrobial action of the films (Gialamas *et al.*, 2010; Sánchez-González *et al.*, 2014).

As mentioned previously the areas of inhibition were significantly wider when the antagonism was detected at 4°C, this may be due to the different growth dynamics of the indicator strain in the presence of bacteriocins and/or better bacterial propagation than the film at 4°C.

In Table 3.3 the results of the evaluation of antimicrobial activity are also summarized against inoculation of *L. innocua* 1776 (~10^5 CFU/cm^2) on the surface of TSA plates which were then covered with FL and FC films and stored for 28 days at 10°C. The results of the enumeration of *L. innocua* showed a significant decrease of the microorganisms identified in the FL film compared to the control film (FC). After only 24 hours, the decrease in *L. innocua* was recorded about two cycles, reaching at the end of the storage period the absence of *L. innocua* at all.

The results confirmed the bactericidal effect of bacteriocin produced by *L. curvatus* 54M16 (Casaburi *et al.*, 2016). While it was also observed that FC film without *L. curvatus* 54M16 did not inhibit *L. innocua*, on the contrary, it increased from 4.44 to 9.20 CFU/cm^2 after 28 days of storage. Sánchez-González *et al*. in 2014 a similar in vitro antimicrobial assay against a strain of *L. innocua* reported a 1.5 log-cycle reduction in microorganisms for membranes containing cells of bacteriocin-producing bacteria (*L. acidophilus* and *L. reuteri*). These films are based on sodium caseinate and methylcellulose compared to control films. Whereas Gialamas *et al.* 2010

reported a 3-log reduction of *L. monocytogenes* in sodium caseinate films containing bacteriocin-producing *L. sakei* cells.

In this study, and as noted in table 3.3, the number of *L. curvatus* remained fairly stable after contact of FL film with the surface of the medium at 10°C during 28 days of storage. Thus, the bacteriocin-producing strain demonstrated the protective effect of the film throughout the storage period.

Avoiding sensory defects is a fundamental challenge in producing dimensionally suitable microcapsules for food application. In a previous study, an advanced live-cell encapsulation methodology was used aimed at ensuring uniform performance (like protection toward cells, exchange metabolites) of the microencapsulated population through the moldable thickness of the encapsulation matrices and the narrow distribution of size and shape along with the smallest achievable microcapsules (De Prisco & Mauriello, 2017).

Theoretically, the number of colonies should remain stable over time because it is assumed that each small microcapsule from which each colony arises (De Prisco *et al.,* 2015). In a previous study (De Prisco & Mauriello, 2017), the bacterial load on microcapsules was determined disrupted and intact, with the main objective of counting the entire encapsulated bacterial population. Whereas, 0.2 M solution of sodium citrate pH 8.00 was used in the same previous study instead of using phosphate buffer as reported in another study (De Prisco *et al.,* 2015) because it was more effective in

dissolving the alginate network also in dairy matrices. All microcapsules showed a high bacterial load, and this is in line with previous experiments that alginate microcapsules carried a high capacity of cells obtained by vibration technique and high encapsulation efficiency close to 100%.

In both previous tables 3.5 and 3.6 are was concluded that the area of inhibition halo was the largest in DM at temperatures 4 and 30 after 144 and 96 days of storage, in both samples MAlg and MAlg + MRS, this indicates higher bacteriocin activity as a result of the higher bacterial load in DM. It was confirmed in the de Prisco study, where the bacterial load was the highest percentage in disrupted compared to Intact (De Prisco & Mauriello, 2017). In Table 3.7, the bacteriocin activity in mMRS and integer microencapsulation samples was limited for 72 hours at 30 °C, but the highest inhibition rate was in the mMRSFree, and this is due to the possibility that the bacterial load rate in it was the highest, and then the antimicrobial activity was the highest.

Probiotic bacteria during food processing and storage, if added to a food matrix, are exposed to many stressful conditions (temperature, osmotic stress, oxidative stress, and pH variations), which ultimately affect their survival (De Prisco & Mauriello, 2017). In Fig. 3.2, antimicrobial activity was observed after 24 h only for samples at pH 4.0 and 6.0 until the end of incubation. Samples at pH 2.0 did not show antimicrobial activity at each time of storage. This indicates that the efficiency and viability of the

54M16 strain-produced bacteriocin, which was released from MAlg + MRS microcapsules, decrease under pH 4.0.

4.2 Conclusion

The film components used in this experiment can serve as efficient carriers for LAB to be used as a biologically active film. Films during storage, obtained in the presence of m-MRS broth showed LAB viability and antimicrobial properties. Nutrient availability influences the viability of LAB and its antimicrobial properties. Compared to control samples, the use of bioactive films against *L. innocua* C6 significantly inhibited the pathogen. As the above results indicated that films bearing *L. curvatus* 54M16 strain can be used to improve food safety as an alternative and effective packaging technology. The second part of the study showed that microencapsulation by vibration technology can produce stable LAB microcapsules with dimensions and morphology suitable for food-based applications, such as bioactive films for food packaging that protect bacteria during food processing, storage, and delivery in foods, that are not normally considered a medium for LAB.

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تطبيق الكبسلة الدقيقة للبكتيريا المُنتِجة للبكتيروسين من أجل تطوير تغليف مضاد للمايكروبات

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الملخص

يهدف هذا البحث إلى دراسة الجدوى والنشاط المضاد للميكروبات لبكتيريا حمض اللاكتيك (LAB) المنتجة للبكتيروسينات المُدمجة في الفيلم الصالح للأكل ودراسة النشاط المضاد للميكروبات للفيلم ضد L. innocua في نظام المختبر. أظهرت نتائج العمل أن الأفلام كانت قادرة على ضمان حيوية عالية للسلالة المُنتِجة للبكتيروسينات (L. curvatus 54M16) عند 4 درجات مئوية خلال 28 يومًا من التخزين. علاوة على ذلك، تم دراسة تأثير الكبسلة الدقيقة على قابلية الحياة والنشاط المضاد للميكروبات لـ LAB لتصميم فيلم صالح للأكل بسلالة مُغلفة في كبسولات دقيقة.