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Efecto de la inmovilización en la producción de una bacteriocina por *Pediococcus Acidilactici* LPS28 aislada de salmón.
(Effect of immobilization on the production of a bacteriocin by the salmon isolate *Pediococcus Acidilactici* LPS28)

Tesis para optar al grado de Doctorado en Ciencias de la Ingeniería con Mención en Ingeniería Química

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Abstract

Lactic acid bacteria (LAB) are use as probiotic microorganism in mammals, fish and fish products, mostly because of production of antagonist substances as lactic acid, acetic acid, oxygen peroxide and bacteriocins. The latter, are known to have a great potential as food bio-preservatives and are used in the production and preservation of food. While immobilization of lactic acid bacteria appears as an alternative to increase both cell concentration and bacteriocin production.

In this study, the salmon isolate *Pediococcus acidilactici* LPS28 was shown to produce an antilisterial bacteriocin. Besides inhibiting the growth of *Listeria* spp. strains, the antimicrobial compound was also active against several strains of lactic acid bacteria but not against staphylococci. The bacteriocin was purified and its size was estimated by Tris-Tricine SDS-PAGE at about 3 kDa and by LC-MS/MS at 2.25 kDa. It was characterized as a heat-resistant (121°C for 15 min) and pH-tolerant peptide, resistant to treatment with trypsin and α-amylase, but not with proteinase K. Antibacterial activity was related to membrane permeabilization. Also, the relationship between microbial growth, bacteriocin production and the effect of fermentation medium using four different growth media: MRS medium, modified MRS medium (mMRS), whey permeate (WP), and lactase-treated whey permeate (LWP), pH and temperature on the occurrence and the maximum levels, to optimize bacteriocin biosynthesis during growth was studied. Either the medium, pH and/ temperature influenced the growth and bacteriocin production in different levels. The higher bacteriocin titers were obtained in LWP broth, at pH 6.0 at 32°C, and the maximum cell concentration was obtained in the same conditions, but at 37°C. The effect of immobilization of *Pediococcus acidilactici* LPS28 entrapped in calcium alginate was studied during fermentation, the effect of the medium and alginate percentage, and the reutilization of the beads. Immobilized *P. acidilactici* LPS28 reached high concentrations in the beads and produced raised bacteriocin titers. Also, better results were obtained in LWP broth at 2% of alginate. The immobilized cells could be reuses up to three times after filtration and resuspension in new medium. *P. acidilactici* LPS28 production kinetics were studied in both planktonic and alginate-immobilized cultures. In planktonic cultures, bacteriocin production paralleled bacterial growth and was optimal in LWP. Upon immobilization, bacteriocin production was still highest in LWP and also was affected by the percentage of alginate

added. In addition, *P. acidilactici* LPS28 displayed poorer growth in the alginate beads than as planktonic cells, but demonstrated an increased bacteriocin production capacity. In addition, several experiments with immobilized cells indicated a shift in bacteriocin activity towards the stationary phase, which may be related to either altered production kinetics or diffusion effects. Also, a continuous fermentation in a glass column reactor was displayed, obtaining bacteriocin titers similar to those in batch fermentation. Finally pediococci and the listeria were inoculated as mono and co-cultures onto smoked salmon fillets at 4°C and 15°C for 24 h. Enumeration of LAB, *L. inoccua* ATCC 33090, and bacteriocin production was performed.

Overall, results indicate that production of bacteriocins may be affected by the culturing method, which may be important when considering food applications in gel-like environments (e.g., cheeses, fermented meats, fish, etc.). Also, they showed that strain *P. acidilactici* LPS28 is a good biopreservative candidate for controlling listerial growth in smoked salmon showing a bacteriostatic mode of action against the target bacteria in the inoculated salmon fillets.

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Chapter I: Introduction

Lactic acid bacteria (LAB) are gram positive bacteria with the ability to produce antimicrobial substances as lactic acid, hydrogen peroxide, acetic acid, carbon dioxide and bacteriocins (Leroy and De Vuyst, 2010). They are use as probiotic microorganism in mammals, fish and fish products (Garriques and Arévalo, 1995, Campaña *et al.*, 2003). Because of production of bacteriocins by LAB, they are known to have a great potential as food bio-preservatives and are used in the production and preservation of food like dairy products, meet and cheese. (Sivakumar *et al.*, 2010). Within lactic acid bacteria, we can found the genus *Pediococcus*, which belongs to the family *Lactobacillacea*. *P.acidilactici* can be part of food fermentations as normal microflora or in starters and have been used in natural and controlled fermentations of vegetables and meat (Piva and Headon, 1994).

Pediocin produced by *Pediococcus* are generally small (< 5kDa) and unmodified peptides known as "antilisterial" or "Listeria-active" peptides. They has received much attention as potential food preservative because of its activity against gram-positive bacteria such as *Staphylococcus aureus*, *Listeria monocytogenes* and *Clostridium perfringens*, as well as gram-negative bacteria such as *Pseudomonas* sps., *Escherichia coli*, *Salmonella typhimurium*, *Aeromonas* and food spoilage bacteria, and its ability to inhibit their growth in several food systems (Pucci *et al.*, 1988; Yousef *et al.*, 1991; Neera *et al.*, 2013). Pediocin PA-1 from *Pediococcus acidilactici* PAC 1.0 is the most studied and already sequenced bacteriocin of this genus of LAB. It is a Class II bacteriocin, with a molecular weight of 4.6 kDa, and it have a very wide range of inhibitory spectrum (Mandal *et al.*, 2008; BACTIBASE, 2015).

Bacteriocin production by *Pediococcus acidilactici* strains can be favored by some growth conditions as incubation conditions like temperature and pH. (Wescombe and Tagg, 2003; Svetoslav *et al.*, 2004; Chang *et al.*, 2007). This species could tolerate a wide range of acidity (Papagianni and Anastasiadou, 2009) only not growing at extreme pH below 3.5. Also pediococci can growth at pH above 8.0 (Zhang *et al.*, 2012). The same author report that *Pediococcus acidilactici* can growth that different temperatures between 25°C and 40°C.

Medium composition also affects the production of bacteriocins and growth of *P. acidilactici* strains (Wescombe and Tagg, 2003; Zalán *et al.*, 2005). Generally, complex medium rich in nitrogen are optimum for the increase of bacteriocin titers by pediococci

(Kemperman *et al.*, 2003; Kawai *et al.*, 2003). The main carbon source used by this strains is glucose, and they are unable to ferment lactose.

Production of bacteriocins is proportional to growth, showing primary metabolite kinetics (Leroy and De Vuyst 2005). Yet, confounding relationships between growth kinetics and bacteriocin production are sometimes found, as bacteriocin release may be limited to specific stages of bacterial population development. This is the case for *Lactobacillus sakei* CTC 494, which only produces sakacin K after a certain minimal cell concentration has been reached (Leroy and De Vuyst 2005). In contrast, enterocin production by *Enterococcus faecium* RZS C5 is restricted to the very early growth phase after which it is switched off (Leroy and De Vuyst 2002). Consequently, bacteriocin activities do not always correlate fully with cell concentration or growth rates of the producer strains and even may improve at suboptimal growth conditions or under stress (Leroy *et al.* 2003; Settani *et al.* 2008). Such observations are likely related to the involvement of inducing factors and quorum-sensing mechanisms (Fontaine *et al.* 2007; Maldonado-Barragán *et al.* 2009; Rizzello *et al.* 2014).

Optimization of bacteriocin generation in food matrices requires a better understanding of the production kinetics and the dependency on process factors (Delgado *et al.* 2007). Yet, most studies have been based on experiments in liquid media with planktonic cells, which differ fundamentally from actual food systems where cells are often growing on solid food particles or in gel-like systems (Antwi *et al.* 2007; Theys *et al.* 2009). Also, bacteriocin production by planktonic cells may be different from the levels obtained with cells growing as compact nests or biofilms on (semi)solid supports, as a result of the involved cell density-related production mechanisms (Braem *et al.* 2014; Rizzello *et al.* 2014). As an example, Cintas *et al.* (1995) found that out of 55 isolates of LAB exhibiting antimicrobial activity on agar media, only 12 of them produced an inhibitory substance in liquid media. Indeed, bacteriocin-driven extermination of neighbour cells to decrease nutrient competition may be more relevant for solid growth than for liquid cultures (Chao and Levin 1981). To date, it is not fully clear if solid growth provokes changes in the expression of relevant genes or if it improves bacterial communication by limited diffusion of signalling compounds (Maldonado-Barragán *et al.* 2009).

Encapsulation of bacterial strains appears as an alternative of gel-like systems to simulate food matrix. Also, it can be use because commonly bacteriocins are produced in stress conditions, as nutrients limitations and overpopulation, which can be generated by encapsulation of the cells (De Souza *et al.*, 2005; Riley and Gordon, 1999; De Vuyst *et al.*, 1996). Furthermore, due to the low survival of the bacteria in the final products, encapsulation allows providing living probiotic cells with a physical barrier that can withstand adverse environmental conditions encountered in the stomach as low pH and unfavourable oxygen conditions (Brusch Brinques *et al.*, 2011; Kailasapathy, 2009; Mandal *et al.*, 2006).

In food biotechnology, encapsulation can be used to wrap or entrap microorganisms, segregating them from the external environment, allowing the release of the cells in the human stomach or intestine at the right time. This technology protects the organism in food during passage through the gastrointestinal tract (Champagne *et al.*, 1994). Other advantages include the prevention of interfacial inactivation, stimulation of production or secretion of secondary metabolites as bacteriocins. In addition, encapsulation may increase the microbial survival and operating efficiency during fermentation (Nazzaro *et al.*, 2011; Nazzaro *et al.*, 2009). Besides increasing the viability of the bacterium, encapsulation facilitates the handling of cells and allows an exchange of substrates on one side and the other product (Rokka and Rantamäki, 2010).

Although no studies of encapsulated bacteriocingenic microoganisms related to the aquaculture industry, had been reported, studies with other bacteria, particularly lactic acid bacteria, had been done. In this investigations it was obtained that bacteriocin production increased up to 50% by encapsulating the microorganism (Ivanova *et al.*, 2002, Nilsang, 2010).

Researchs in aquatic organisms have shown that the production and diversity of bacteriocins in the aquatic environment is abundant (Bagenda et al, 2008). It has also been shown that purified bacteriocins not affect the sensory qualities of seafood and that are stable at salinity concentrations. Brillet *et al.* (2005) demonstrated that bacteriocin-producing bacteria *Carnobacterium divergens* V41 can be used as a bio preservative to inhibit the growth of Listeria monocytogenes in salmon. It has also been suggested that the bacteriocins can be combined with some methods for antimicrobial treatment and preservation to produce

synergistic effects, as the incorporation of bacteriocins in bioactive packaging (Calo-Mata *et al.*, 2007; Pilet and Leroi, 2011). Bacteriocins encapsulation for biopreservation could reduce packaging costs due to low cost and antibacterial amount needed to bind to gel (Galvez *et al.*, 2008). Therefore, immobilized lactic acid bacteria producing a bacteriocin and the combination with methods used in the aquaculture industry can has the potential to increase the guarantee of freshness ensuring inhibition of microorganisms that cause deterioration.

Some studies (Barbosa et al, 2014; Nazzaro *et al.*, 2012; Sarika *et al.*, 2012; Nilsang, 2010; Rao *et al.*, 2008; Idris and Suzana, 2006; Narita *et al.*, 2004; Ivanova *et al.*, 2002; Wan *et al.*, 1995) had studied the effect of encapsulation either on bacteria growth, lactic acid production and least bacteriocin production. The results obtained are different, but some of them reported higher bacteriocin titers by bacteria encapsulated than cultivated as free cells (Barbosa et al, 2014; Nilsang, 2010; Ivanova *et al.*, 2002).

Batch fermentation is the most common approach to bacteriocin production but this is often unsuitable for industrial application (Liao *et al.*, 1993; De Vuyst and Vandamme, 1991). Continuous fermentation, with a continuous flow of unfermented medium into the fermenter and a corresponding continuous flow of fermented product out the system, offers important advantages over conventional batch processes, such as higher conversion rates, faster fermentation rates, improved product consistency, reduced product losses and environmental advantages (Verbelen *et al.*, 2006). But, in the case of free-cell continuous fermentation, it is limited by cell wash out (Liu *et al.*, 2005). As an alternative, continuous fermentation with immobilized bacteriocin-producing strains appears.

Few research is available regarding continuous fermentation for production of bacteriocin using immobilized bacteria. Nisin production was studied in batch culture using *L. lactis* subsp. *lactis* NZ1 immobilized in coated alginate beads (Zezza *et al.*, 1993) and in continuous fermentation for brevicin, nisin, pediocin (Wan *et al.*, 1995), and divercin (Bhugaloo-Vial *et al.*, 1997). Huang *et al.* (1996) immobilized *Pediococcus acidilactici* UL5 in k-carrageenan and obtained an increase on pediocin 5 production. Others, like Cho *et al.*, (1996) described a packed bed bioreactor capable of stable long-term production of pediocin using *Pediococcus acidilactici* PO2, and Yang and Ray (1994) found that high cell density in the fermenter under uncontrolled pH are favorable for production of pediocin AcH by *P. acidilactici* LB42923.

Finally, although bacteriocinogenic strains of LAB show interesting potential as starter cultures or bioprotective cultures for the food industry (Leroy and De Vuyst, 2004), challenge tests using co-cultures remain always needed to explore their *in situ* activity in actual food products. A co-culture is a cell cultivation set-up, in which two or more different populations of cells are grown with some degree of contact between them, for instance using a bacteriocin producer and a target bacterium (Goers *et al.*, 2014). Several studies have indeed indicated that LAB cultures are able to produce their bacteriocins in food matrices, and consequently display inhibitory activity towards added populations of sensitive food spoilage or pathogenic bacteria (Leroy and De Vuyst, 2007). As representative examples, co-cultures of LAB and pathogenic bacteria have been investigated in sourdough, fermented sausage, and cheese environments (*e.g.*, Leroy *et al.*, 2005; Foulquié Moreno *et al.*, 2003), and also in smoked salmon (*e.g.*, Todorov *et al.*, 2011; Tahiri *et al.*, 2009).

In this study was initiated to evaluate the production of an antilisterial bacteriocin by the salmon isolate *Pediococcus acidilactici* LPS28, and to study the effect of alginate immobilization on cell growth and bacteriocin production. The latter, because fish and fish products usually are affected by the spoilage bacteria *Listeria monocytogenes*, so the study of a natural protein that can act against it under different culture conditions is very important in food industry. Also, immobilizing producer cells in alginate matrices offers the potential to study bacteriocin production in gel-like systems, offering a better simulation of the texture of, for instance, meat, fish, and cheese matrices, than would be the case in liquid media. Besides, alginate systems may also offer interesting technology for the industrial production of bacteriocins as food additives (Ivanova *et al.* 2000).

Purpose of the study:

The present study aims to:

- Characterized and purified a bacteriocin produced by salmon isolated *Pediococcus acidilactici* LPS28.
- Evaluate the effect of temperature and medium composition on bacterial growth and bacteriocin production by *P. acidilactici* LPS28, cultivated as planktonic cells.
- Study the effect of encapsulation of *Pediococcus acidilactici* LPS28, on the production of an antimicrobial peptide.

- Evaluate the medium composition and alginate concentration on bacterial growth and bacteriocin production by *P. acidilactici* LPS28, cultivated as immobilized cells.
- Elucidate the kinetics of cell growth and bacteriocin production of a *Pediococcus acidilactici* strain (LPS28), and their dependency on growth in liquid or solid media, using alginate.
- Investigate feasibility of producing a bacteriocin continuously, using calcium-alginate immobilized *Pediococcus acidilactici* LPS28.
- Investigate growth and pediocin production by *Pediococcus acidilactici* LPS28 and the resulting inhibitory action on added *Listeria innocua* ATCC 33090 in smoked salmon.

Hypotheses of the study

- ***Pediococcus acidilactici* LPS28 produces a bacteriocin with antilisterial properties.** Pediocin will be part of class II bacteriocins.
- **Temperature and medium composition will present an important effect con pedioccoci growth and bacteriocin production.** The optimum temperature and medium composition for cell growth could differ from the optimum for the peptide production.
- **By encapsulating the biomass concentration increases, and thus a stress is generated between bacteria that enables increased production of bacteriocins.** Also, the increase in the production can be generated because the encapsulation matrix acts as protection and separate the bacteriocins, which are an extracellular metabolite that diffuses out of the capsules, of proteolytic enzymes released by bacteria that remain within the spheres.

Limitations of the study

- No bacteriocin sequencing had been done.

Chapter 2: Literature Review

2.1. Lactic acid bacteria in fish

Lactic acid bacteria (LAB) are characterized as Gram positive bacteria, not moving, not sporing bacteria, and are generally catalase negative and devoid of cytochromes. Produce lactic acid as the major or sole product of fermentative metabolism (Salminen *et al.*, 2004; Mozzi *et al.*, 2010). LAB have high nutritional requirements for its development, requiring carbohydrates, amino acids, peptides, nucleic acids and vitamins for its growth. Within this group, relatedly to taxonomy, we can find more than 20 genus, between bacillary (lactobacillus and carnobacteria) and coccoid bacteria (streptococci), being the principal associated to food technology: *Aerococcus*, *Canobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, and *Weisella* (Salminen *et al.*, 2004).

LAB species adapt to various environmental conditions, and can be found widely in nature. Commonly, we can find lactic acid bacteria in the gastrointestinal tract of several endemic animals such as mice, pigs, humans, among others, in milk or dairy products, seafood, and on the surface of some plants. LAB are use in the production and preservation of food as cheese, meat and yogurt. Also, they are use as probiotic microorganisms in humans, mammals and recently in fish and crustaceous (Garriques and Arévalo, 1995, Campaña *et al.*, 2003). The latter, mostly because of their capacity to produce antimicrobial substances as lactic acid, hydrogen peroxide, carbon dioxide and bacteriocins (Vaughan *et al.*, 2005).

In fish, commonly bacteria is find in the skin, mucus, gills and guts. The microorganisms vary depending on the fish species and ambient parameters, especially temperature. Some studies had demonstrate that lactic acid bacteria are part of the normal microbiota of the digestive tract on fish. Some of species and genus that had been reported are: *Lactobacillus plantarum*, *Carnobacterium* ssp., *Streptococcus* ssp., *Leuconostoc* ssp., *Lactobacillus lactis*, and others (Ringo and Gatesoupe, 1998; Joborn *et al.*, 1999).

Several studies had reported had LAB had positive effects on growth, survival and pathogens elimination on aquatics organisms. However, as the diversity of inhibitory compounds produced by LAB is wide, strict studies are needed on mode of action of this on

different environmental conditions to their origin, because in most of the cases, strains isolated from human or mammals had been used instead of microorganisms isolated from aquatic species (O´Sullivan *et al.*, 2002).

2.2. Bacteriocins

Bacteriocins are antimicrobial peptides with different levels and spectrum of activity, mechanism of action, molecular weight and psychochemical properties (Stoyanova *et al.*, 2012). They could be synthetized by both Gram positive and negative bacteria (Jeevaratham *et al.*, 2005). However, bacteriocins produced by lactic acid bacteria have been of great interest on food industry because of: are produced by commercial LAB (lactococci, lactobacillus, pediococci), are considered safe for consumption, are not toxic for eukaryote cells and showed an inhibition spectrum wider than bacteriocins from Gram negative bacteria (Nes *et al.*, 2007).

Bacteriocins are secondary metabolites of LAB, define as products of ribosomal synthesis of about 20 and 60 aminoacids. They are secreted extracellularly and presents high bactericidal or bacteriostatic activity against other strains, bacteria related to the producer microorganism, pathogens and food spoilage bacteria (Eijsink *et al.*, 1998; Cotter *et al.*, 2005).

The production of bacteriocins occurs in the logarithmic phase of the bacterial development or at the end of this one, with a direct relation with the produced biomass. Between the principal characteristics of this peptides, we can find that they are heat and pH stable (Chen and Hoover, 2003). However, Jack *et al.*(1995) showed that bacteriocins are destroyed at pH higher than 10. The stability to high temperature it may be due the formation of small globular structures, the presence of hydrophobic regions or the formation of stables crosslinking (Alquicira, 2006). Also, they are inactivated by proteases (Quintero, 2006).

The inhibition spectrum include the pathogens *Clostridium botulinum*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella*, *Escherichia coli*, and some *Bacillus* species (Wu *et al.*, 2004; Jeevaratnam *et al.*, 2005).

Bacteriocins from LAB are classified between 3 principal groups (Savadogo *et al.*, 2006; De Vuyst and Leroy, 2007; Beshkova and Frengova, 2012). Class I (Lantibiotics), Class II (No Lantibiotics) and Class III (Thermolabile). The first include small peptides of

19-30 aminoacids, policycle, with a molecular weight below 5kDa, with low heat stability and modified postranslationally (Riley and Wertz, 2002; Savadogo *et al.*, 2006; Montalbán-López *et al.*, 2011). Class II include bacteriocins of 30 to 60 aminoacids with a molecular weight below 10 kDa. They are heat and pH stable (Montalbán-López *et al.*, 2011; Beshkova and Frengova, 2012). Finally, bacteriocins from Class II are also called bacteriolisins, and include peptide with a molecular weight higher than 30 kDa that are heat labile (Abriouel *et al.*, 2011).

Most studies had demonstrate that the bactericide activity of bacteriocins is mainly against Gram positive bacteria (Jack *et al.*, 1995). Nevertheless, exists many bacteriocins that presents a wide rank of action, inhibiting Gram negative bacteria (Mota-Neira *et al.*, 2000; Chung and Yousef, 2005; Gautam and Sharma, 2009), pathogens fungi (Ennahar *et al.*, 2000) and viruses (Jenssen *et al.*, 2006). Cotter *et al.*, (2005) showed that bacteriocins of Gram positive bacteria presents different mode of action respect to their classification. The mode of action of lantibiotics is related to the destabilization (due to pore formation) of the citoplasmatic membrane. No-lantibiotics peptides presents a structure helical amphiphilic that allows them to insert in the cells membrane, generating its dead. Class III bacteriocins, acts directly on the cell wall of Gram positive bacteria producing cell dead and lisis (Cotter *et al.*, 2005).

There is a data base of the bacteriocins characterized and sequenced till the date called BACTIBASE. The web page contains 177 bacteriocin sequences, of which 156 are the products of Gram-positive organisms and 18 of Gram-negative organismsThe database now comprises 31 genera, as shown in Figure 1. Without surprise, the lactic acid bacteria (order Lactobacillales) make up the predominant group of producers, with 113 bacteriocins (Bactibase, 2016). Also, in the statics section of the page, there is physicochemical, structural and taxonomic information of the peptides. Figure 1 shows the available information for pediocin PA-1.

Nisin, is the most representative and studied bacteriocin at the moment. It was described in 1928, and it was the first bacteriocin isolated from lactic acid bacteria (*Lactococcus lactis lactis*). It is the best characterized bacteriocin and it is used as food preservative. Also, it is recognized by the FDA (Food and Drug Administration) as GRAS (Generally recognized as safe). It is naturally produced in some dairy products and it is used

in food production and as additive on this products to prevent decomposition produced by bacteria. Other well characterized bacteriocins are pediocin PA-1, plantaricin E/F and J/K, divergecin A, helveticin J, among others (Monroy *et al.*, 2009).

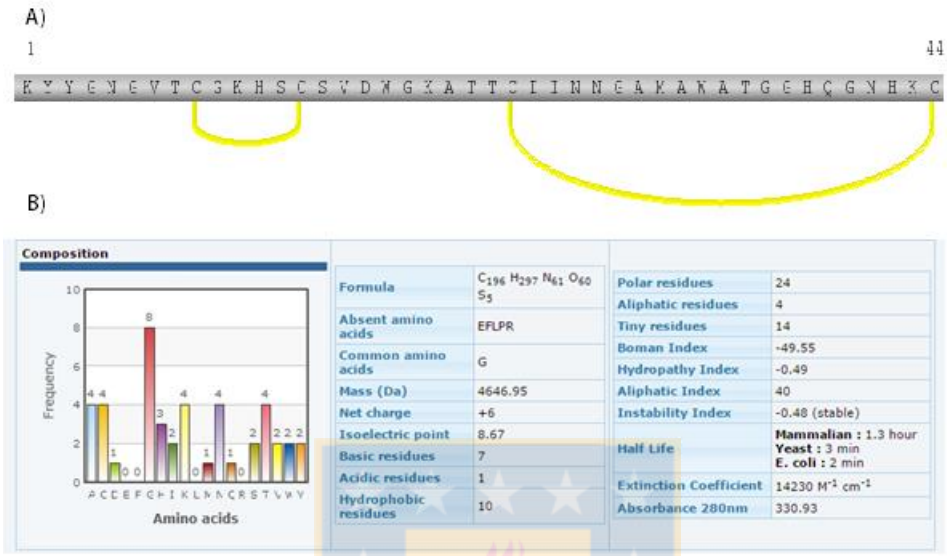

Figure 1. Structure of pediocin PA-1(A) and physicochemical information of pediocin PA-1 (B)(Bactibase, 2016).

Bacteriocin production can be quantified as the specific production in AU/g biomass, therefore arbitrary units of bacteriocins per gram of biomass. Leroy and De Vuyst (1999), Cheigh *et al.*, (2002) and Sudirman *et al.*, (1994) demonstrated that the maximum bacteriocin production, after 24 h, vary depending on the producer microorganism and its growth conditions, and it is in the range of 290 and 8010 kAU/g biomass.

It is important to consider the growth conditions of the producing bacterium, as studies have shown that the production of certain bacteriocins can be favored under certain growth conditions, such as temperature, pH and the composition of the culture medium, being specific to each microorganism producer (Monroy *et al.*, 2009).

2.3 Bacteriocins of aquatic bacteria

Bacteriocins produced by aquatic bacteria have generated great interest due to their potential use as probiotics or natural antibiotics in seafood industry. The first bacteriocin

isolated from a microorganism of this habitat was detected in *Vibrio harveyi*, named haveycin. Bacteriocins isolated from aquatic organisms are diverse, share common characteristics with bacteriocins of bacteria and archaea. They can be small peptides (5-10 kDa) as microcinas of Gram negative bacteria, microhalocinas of halobacteria, and Class I and II of Gram positive bacteria. They may also have a larger size (10-90 kDa) as colicins from Gram negative bacteria. To the date, there are more than 12 bacteriocins isolated from aquatic microorganisms, and many more peptides with similar characteristics that bacteriocins, being most isolated from bacteria of the genus *Carnobacterium*, *Enterococcus* and *Streptococcus* (Bakkal *et al.*, 2012;. Pilet and Leroi, 2011). Most of the bacteriocins produced by lactic acid bacteria isolated from fish and shellfish are active only against Gram positive bacterias.

2.4. Encapsulation of bacteriocin producer microorganisms

One of the biggest problems associated with the production of bacteriocins is the low yield obtained when the cells are grown freely (Ivanova *et al.*, 2002). Therefore, recent studies are based on increasing both cell concentration and bacteriocins production. One of the methods to obtain this, is bacteria encapsulation. As bacteriocins are commonly produced under stress conditions, such as nutrient limitation and overpopulation, which is generated by encapsulating bacterial cells (Riley and Gordon, 1999). Furthermore, due to the low survival of the bacteria in the final products, encapsulation allows providing living probiotic cells with a physical barrier that can withstand adverse environmental conditions encountered in the stomach as unfavorable low pH and oxygen conditions (Kailasapathy, 2009).

Encapsulation can be defined as a process by which small pieces of an active ingredient of gas, liquid, or a solid are packaged within a second material in order to protect it from the surrounding environment (Anal and Singh, 2007).

Encapsulation aims to protect an unstable nucleus (or active compound, in this case microorganisms that produced bacteriocins) from their environment, improving stability, extending the core life and giving a sustained and controlled release. The structure formed by the encapsulating agent around the center of the substance is known as wall or matrix. The properties of the wall are designed to protect the center and release it under specified conditions while allowing the exchange of small substances through the membrane (Shahidi

and Han, 1993; Franjione and Vasishtha, 1995). Encapsulation of cells offers space for cell growth and good diffusion properties. Both external and internal mass transfer in the gel influences the transport speed of substrate and product particles (Ivanova *et al.*, 2002).

In food biotechnology, encapsulation can be used to wrap or entrap microorganisms, segregating them from the external environment with hydrocolloid covers, allowing the release of the cells in the human stomach or intestine at the right time. This technology protects the organism in food during the passage through the gastrointestinal tract (Champagne *et al.*, 1994). Other advantages include the prevention of interfacial inactivation, and stimulation of production or secretion of secondary metabolites as bacteriocins. In addition, encapsulation may increase microbial survival and operating efficiency during fermentation (Nazzaro *et al.*, 2009). Important to note that the particles should be insoluble in water to maintain structural integrity in food matrix and upper gastrointestinal tract, and also the properties of the particles should permit gradual release of cells during intestinal phase (Nazzaro *et al.*, 2011) and protect the active content from environmental stress like acidity, oxygen and gastric conditions. Besides increasing the viability of the bacterium, encapsulation facilitates the handling of cells and allows an exchange on one side to the other of substrates and products (Rokka and Rantamäki, 2010).

2.4.1. Materials used in microorganisms encapsulation

Materials or food contact matrices are usually natural compounds, but mostly be generally recognized as safe (Generally recognized as safe, GRAS) for human health. For the encapsulation of microorganisms, the most widely used natural polymers should be biocompatible and GRAS. Among the most common are alginate, K-carrageenan, chitosan, milk protein, pectin, starch, and poly-l-lysine (Nazzaro *et al.*, 2011), being the first used in this study.

Alginate is a hydrocolloid which is able to absorb water, has easy handling and its safety. It also has gelling, stabilizing and thickening properties, that's why it has been of great interest to the food industry. The alginate is described as a polyionic and hydrophilic linear polysaccharide from seaweed consisting of two monómetos in their structure, α-L-guluronic (G) acid and β-D-mannuronic (M) acid which are distributed in sections constituting homopolymers or heteropolymers (Figure 2). Both the distribution of the monomers in the

polymer chain and the volume of the carboxyl groups give the gel formed characteristics of rigidity or flexibility depending on the content of G. The gelling process occurs in the presence of multivalent cations (except magnesium) where calcium ion is the most used in food industry. The capsules are porous, and permit diffusion of water into and out of the matrix (Rokka and Rantamäki, 2010). Alginate encapsulation is widely used in laboratory scale, and their use is preferred in encapsulation of probiotics because of their simplicity, biocompatibility, nontoxicity, and low cost (Krasaekoop *et al.*, 2003). One of the advantages of using alginate is that protects the encapsulated compounds of adverse factors such as heat and moisture, thereby enhancing stability and bioavailability. Among its disadvantages is the fact they are very sensitive to an acidic environment, which is not compatible for use in the acidic conditions of the stomach. This disadvantage can be offset by mixing alginate with other polymeric components, covering capsules or applying a structural modification of alginate using different additives.

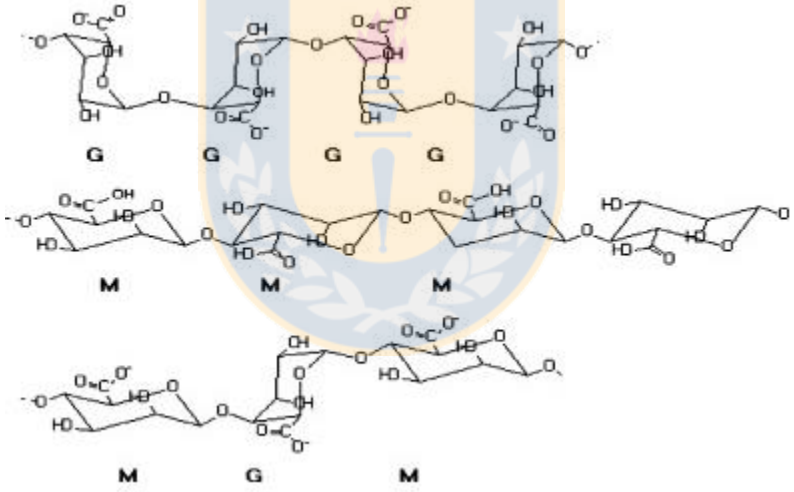

Figure 2. Alginate components: M-M, G-G, and blocks of M-H (Hian *et al.*, 2012)

2.4.2. Microorganisms encapsulation methods in calcium alginate

Before working with calcium alginate is important to know the gelling process, which starts from a solution of alginate salt and an external or internal calcium sort from which calcium ion diffuses to reach the polymer chain generating a space structural rearrangement resulting in a solid material with characteristics of a gel. Ionic gelation mechanisms have been carried out mainly by two processes: gelation internal and external. Internal gelling is the controlled release of calcium ion from an internal source of insoluble or partially soluble

calcium salt dispersed in the solution of sodium alginate. In this case, the calcium ion release depends on the salt solubility and media pH. Regarding the external gelation, it is the spread of the calcium ion from a source surrounding the hydrocolloid to the alginate solution at neutral pH. Gel formation starts at the interface and moves inwardly as the surface is saturated with calcium ions, so that the sodium ion from the alginate salt is displaced by the divalent cation solubilized in water (Lupo and al., 2012). Both processes are shown in Figure 3.

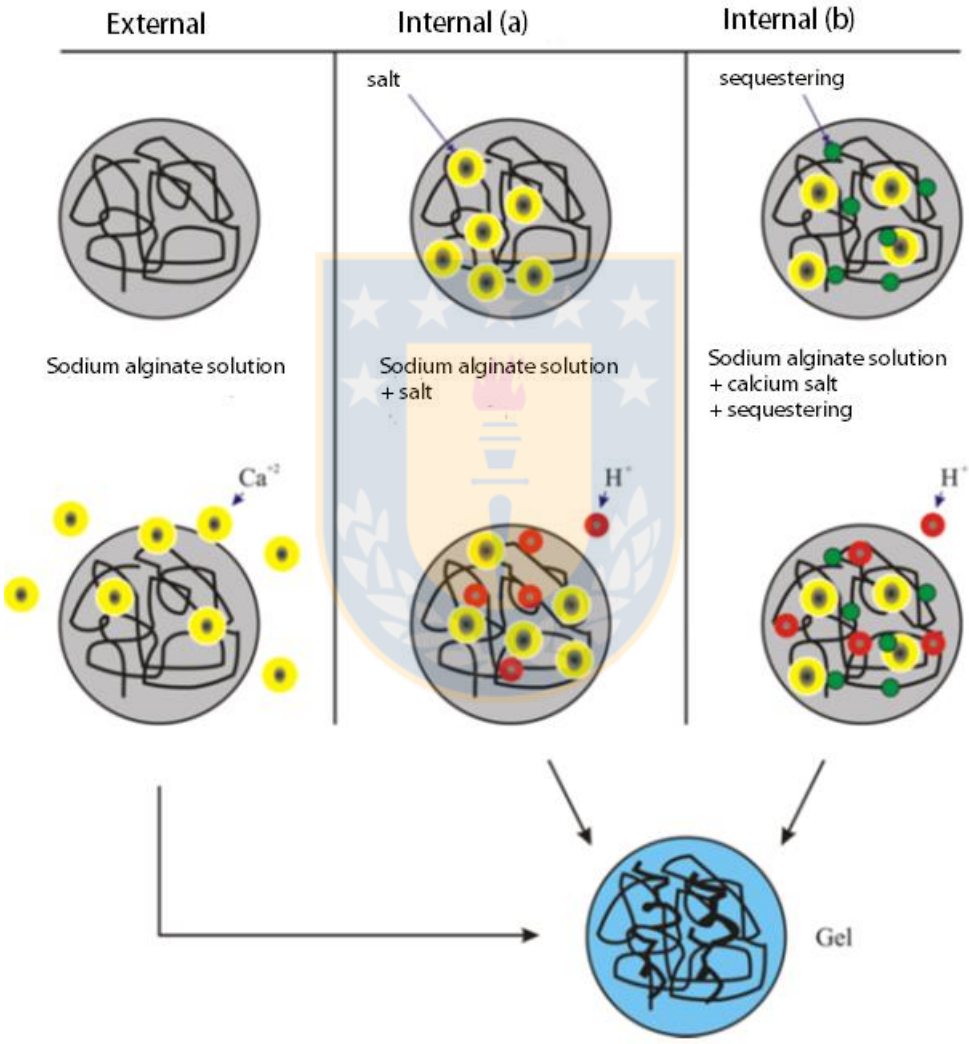

Figure 3. Ionic gelification mechanisms. A. insoluble salt. B. partially soluble salt (Modified from Lupo *et al.*, 2012)

Depending on the encapsulation technique used, the different gelation mechanisms mentioned are observed. The main techniques used with calcium alginate are extrusion and emulsification.

Extrusion is a physical technique to encapsulate probiotic cells and hydrocolloids are used as encapsulation materials. It consists on projecting the solution containing the microorganisms with a high pressure nozzle. A solution with encapsulating material is prepared, the probiotic cells are added, and the solution is atomized by a nozzle. The extrusion method is simple and inexpensive and uses a smooth operation that does not cause damage to cells and provides a high viability of probiotics (Kraseakoopt *et al.*, 2003). A disadvantage of the method is the difficulty of large scale production because capsule formation is one by one which brings as a consequence long gelation times (Mofidi *et al.*, 2000). Additionally, it is considered aspects influencing its spherical shape and size as the spacing of the nozzle to the bath, the effect of gravity and surface tension of the solution that induces gelation (Chan *et al.*, 2009). Despite all these factors, extrusion encapsulation technique has been traditionally used due the production of capsules with uniform sizes. Therefore, in extrusion method, the gelation occurs via external gelling as alginate droplets fall on the source containing the divalent ion.

Emulsification is a chemical technique to encapsulate living and probiotic cells that also uses hydrocolloids as encapsulating materials. The principle of this technique is based on the relationship between continuous and discontinuous phases, where a small volume of a cell suspension-polymer (discontinuous phase) is added to a larger volume of a vegetable oil (continuous phase), which can be soybean, sunflower, canola or corn oil. The mixture is homogenized to form a water in oil emulsion. Once formed the emulsion, the water-soluble polymer must be insolubilized to form small gel particles in the oil phase (Krasaekoopt *et al.*, 2003). Then, the spheres are collected by filtration. In the emulsification technique, the alginate is added prior to the emulsification. The addition of an oil soluble acid such as acetic acid reduces the pH of alginate 7.5 to about 6.5, initiating gel formation with Ca 2+ ions.

Preparing capsules by emulsification can be carried using external or internal gelation, where the first is based on the liberation of calcium ion from an insoluble or partially soluble complex in which case a sequestering agent is added (for example gluconolactano or EDTA) contained in a solution of alginate-component which is dispersed in a non-aqueous continuous phase generating a water in oil emulsion (Gouin, 2004); and external gelation occurs with the dispersion of a mixture component-alginate solution in a non-aqueous continuous phase, followed by the addition of a calcium source that when it diffuses to the

dispersed phase, gelation starts allowing encapsulation and destabilization emulsion for separating the capsules formed (Lupo *et al.*, 2012).

Emulsion technique is easy to scale and gives high rates of bacterial survival (Chen and Chen, 2007). The size of the spheres is controlled by the stirring speed, which can range from 25μm to 2mm. Important to note that the reaction time affects the formation of the capsules and also the survival of microorganisms (Yañez *et al.*, 2008). This methodology is used to improve productivity in the fermentation, that is, increase the bacterial concentration during the fermentation process (Yañez *et al.*, 2008).

2.5. Culture of encapsulated lactic acid bacteria producing bacteriocins

In 1995, Wan *et al.*, studied the continuously production of three different bacteriocins (pediocin, brevecin and nisin) using bacteria encapsulated in calcium alginate. They observed bacteriocin production, glucose consumption and repetitive batch fermentation of 24 h changing the culture medium. For continuous fermentation trials with immobilized cells they used a column of 2.5 cm long and 1.3 cm wide, without temperature control. The concentrations of viable cells, glucose, pH and activity of the bacteriocins were measured from the effluent. They obtained that only brevecin production increased (by 150%) with respect to assays of planktonic cells fermentation, since the production of nisin and pediocin didn't vary. On the other hand, they achieved that the alginate beads were stable to repetitive batch fermentation as well as in the continuous fermentation. Regarding the production of bacteriocins, which was obtained during the first cycle in discontinuous operation of the encapsulated spheres was lower than in subsequent cycles. In addition, they obtained the glucose consumption was not full and there was a 1.9% uneaten.

Ivanova *et al.* (2002) observed the difference in the production of bacteriocins in a batch process with planktonic cells with the production by bacteria encapsulated in calcium alginate in the same type of fermentation, using a 250 mL Erlenmeyer flask. They obtained that when the bacteria were grown in free form, the production of bacteriocins was maximum during the fermentation of 48 h, and then begin to decrease significantly in the culture medium, which can be explained due to digestion of bacteriocins by proteolytic enzymes system cells after cell death. Also they report that when bacteria are encapsulated, the concentration of bacteriocins obtained was 4 times greater. During fermentation, the

concentration of bacteriocins by immobilized bacteria increased rapidly and peaked after 72 h of cultivation. Bacteriocin production was observed throughout the fermentation (96 h). This is due to the protective role of alginate beads separating bacteriocins in the culture of proteolytic enzymes remaining in the spheres. Furthermore, the authors observed how the production of bacteriocins was maintained during cycles, changing the culture media every 96 h, obtaining that the protein concentration was the same in the first cycle and in the subsequent two cycles. Also, the viability of the bacteria was also studied in the spheres, obtaining that they remain stable throughout the fermentation when they are immobilized.

Nilsang, meanwhile, in 2010, studied three different lactic acid bacteria. In the study, the bacteria were encapsulated in calcium alginate and also cultured as planktonic cells in batch processes in a fermenter of 750 ml for 24 h. It was reported that glucose uptake was similar in all cases, not consumed completely at the end of fermentation. The lowest bacteriocins concentration was obtained for plankonics cells grown in batch fermentation under stirring. Fermentation of planktonic and encapsulated cells without stirring presented higher protein titers. Furthermore, production of bacteriocin by encapsulated bacteria was 4 times greater than when the bacteria were grown freely.

No studies where the effects of alginate concentration and the diameter of the spheres in bacteriocin production exists, only in lactic acid production. Idris and Suzana (2006), studied five different concentrations of alginate from 1% to 8%. The higher lactic acid production was obtained at a concentration of the alginate spheres of 2%. Moreover, they added that the consumption of glucose by immobilized bacteria in calcium alginate at 2% was higher than in the other percentages. This was due that at very low concentrations of alginate, the spheres obtained were very soft so they break easily, generating the release of bacteria. At alginate concentrations higher than 2%, very hard spheres were obtained, causing nutrient diffusion problems. This can be related to bacteriocins production, since problems generated also influence the production of the proteins. With respect to the diameter of the spheres, the greater production of lactic acid obtained was with a diameter of 1 mm. By increasing the diameter of the spheres to 2 mm and 5 mm, the lactic acid production significantly decreased which is attributed to an increase in the volume / surface ratio. Important to note that the studies were conducted in a batch fermentation with a volume of 250 ml.

2.6. Mass transfer for immobilized cells

By studying the immobilization of microorganisms it is necessary to delve into issues related to mass transfer in bioreactors with immobilized cells and the diffusivity of the compounds that diffuse into the immobilization matrix and products diffuse into the liquid phase. So it is necessary to find a way to get data from both mass transfer in systems with immobilized bacteria and transport parameters of the compounds to and from bacteria.

With respect to mass transfer, the effect of immobilization in it is critical, as well as cellular metabolism. In these cases, the external mass transfer involves the transfer of nutrients from the bulk of the liquid phase to the surface of the capsule containing the bacteria, while the internal mass transfer involves the transfer of substrates and products within it, and to inside and outside the cells. Metabolite concentration in the immediate vicinity of cells can be altered in immobilized cell systems due to limitations in internal and external mass transfer. Such changes may force the cells to alter their metabolic status and thus affect the efficiency and quality of the fermentation process. Therefore, considering the fermentation using immobilized cells, it must take into account the changes that are generated for bioreactor design and operation, so it is necessary to optimize these steps, as well as the method of immobilization and the fermentation conditions.

In the case of immobilized bacteria that produce a metabolite, the substrate must pass through the bulk of the liquid medium, the outer liquid film surrounding the immobilized particle, the liquid-solid interface, liquid into the solid gel phase, the resistance caused by the formation of microcolonies and finally it must enter the bacterial cells where the reactions occur, as observed in Figure 4.

The external mass transfer, meanwhile, refers to the transport of nutrients from within the bulk to the outer liquid film surrounding the immobilized cells, and is characterized by the mass transfer coefficient in the liquid film, k_L.

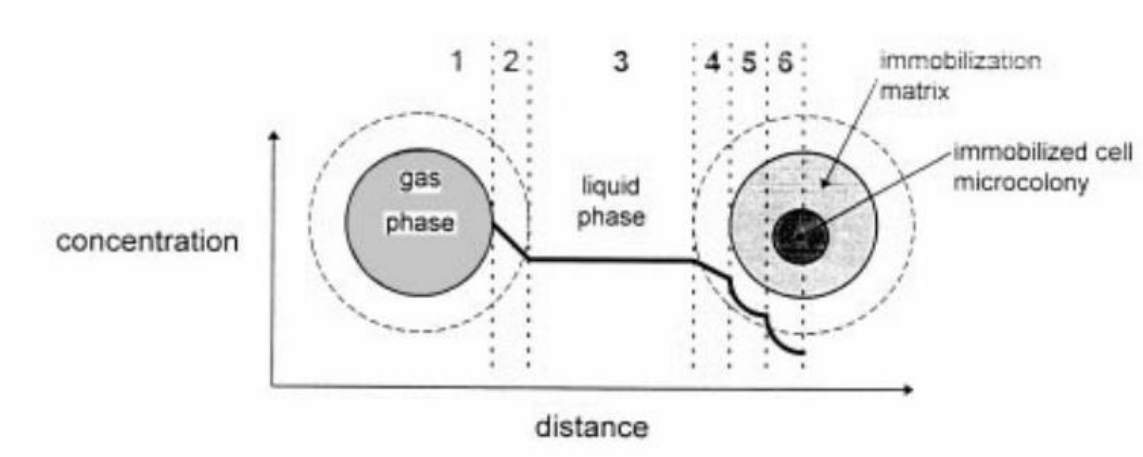

Figure 4. Transport of oxygen from the gas phase to an immobilized cell matrix; 1, gas phase; 2, liquid film; 3, bulk liquid phase; 4, liquid film; 5, solid matrix; 6, microcolony containing cells. (Pilkington et al., 1998)

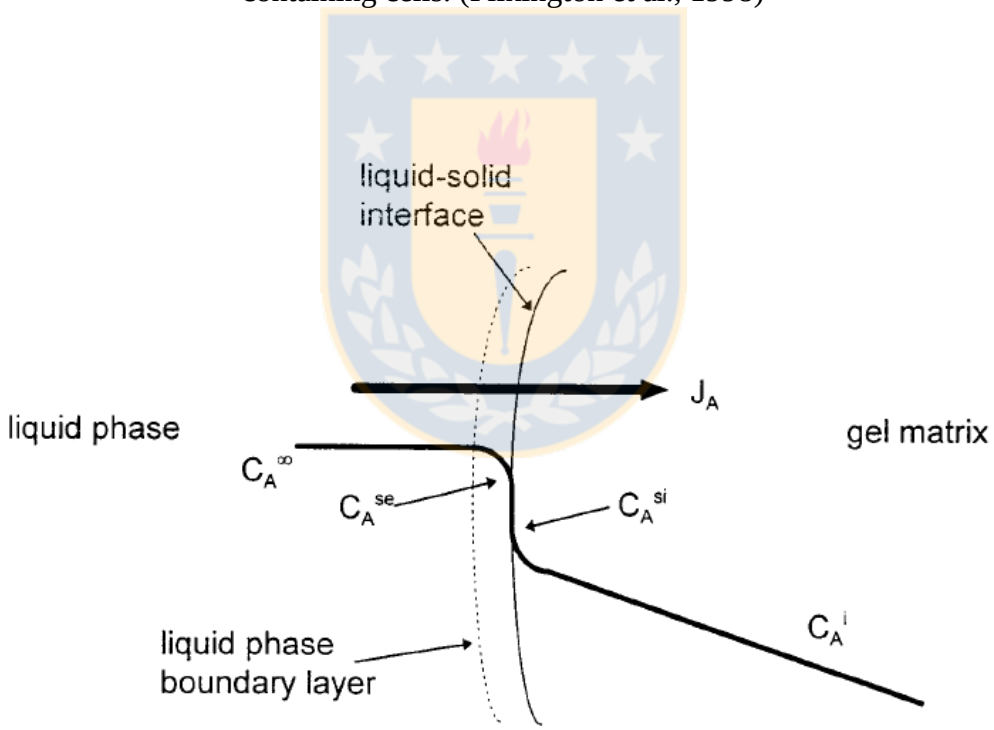

Figure 5. Concentration profiles for overall mass transfer of substrate from the bulk liquid phase into a gel bead. (Pilkington *et al.*, 1998)

Furthermore, Figure 5 describes the flow of a substrate, J_A, to the surface of the sphere containing the immobilized bacteria. It shows that J_A depends on k_L and on the substrate

concentration in the liquid bulk and the substrate concentration in the particle surface, as described by equation (1). In this case the flux per unit of area perpendicular to the surface of the sphere is obtained, and the driving force for mass transfer $(C_A{}^{\infty} - C_A{}^{se})$, can be extracted.

$$J_A = \left({}^{D_L}/_{\delta}\right)(C_A{}^{\infty} - C_A{}^{se}) = k_L(C_A{}^{\infty} - C_A{}^{se}) \qquad (1)$$

Where $C_A{}^{\infty}$ is the substrate concentration in the bulk, and $C_A{}^{se}$ is the external concentration of the substrate on the surface of the particle.

It is noteworthy that the reactor type, size and operating conditions are factors to consider as they act directly on the external mass transfer in immobilized cells, affecting the value of k_L.

The internal mass transfer in the beads, which allows the transfer of substrates and products to them, can be studied through the Fick's law, if it is considered that this occurs only by diffusion. This law is expressed by equation (2), for spherical particles, in radial coordinates, where J is the rate of mass transfer of species per unit of area, C_L is the solute concentration in liquid phase in the pores, r is the radius, and D_e is the effective diffusion coefficient of species A in the polymer matrix, and rp is the radius of the particle.

$$J(C_L) = -D_e\left({}^{\partial C_L}/_{\partial r}\right)_{rp} \qquad (2)$$

Furthermore, the value of D_e is obtained experimentally, and for 2% calcium alginate gels using glucose as substrate is close to 6.0 x 10^{-6} cm^2/s (Pilkington *et al.*, 1998).

It is important to note that studies have shown that bacteria are confined in a layer on the periphery of the trapping gel matrices due to the lack of substrates in the center of the spheres (Lewandowski *et al.*, 1993). The internal mass transfer can be optimized by adjusting the size, texture and porosity of the matrix, where a smaller size presents minor limitations on mass transfer.

Concerning the design of reactors for immobilized cells, the selection of an appropriate design of a reactor of immobilized cells is critical to the success of the process. In general, the type of reactor chosen determines the type of immobilized cells that can be used and vice versa. Some preparations of immobilized cells can only be used in one type of reactor, while inorganic, porous and spherical spheres, can thrive in various types of reactors. Above, depending on the physical and biological properties of immobilized cells (Tapion and Tampion, 1987).

According to Zayed and Winter (1995), Schepers *et al.*(2006), Wan *et al.*(1995) and Liu *et al.*(2005), results in continuous fermentations with respect to production of bacteriocins or lactic acid, and the bacterial growth and the stability are better than in fermentations discontinuous. Liu *et al.*(2005) and Wan *et al.*(1995), used a packed bed reactor, which had being the most studied type for fermentation of immobilized bacteria.

2.7. Potential use of encapsulated lactic acid bacteria producers of bacteriocins in the aquaculture industry.

No recent studies exist on encapsulated microorganisms related to the aquaculture industry that is produce bacteriocins, only reports have been conducted with other bacteria, particularly lactic acid bacteria. In investigations it was obtained that bacteriocin production was increased up to 50% by encapsulating the microorganism (Ivanova *et al.*, 2002, Nilsang, 2010). One of the hypotheses to demonstrate this fact is that by encapsulating the cells, the biomass concentration increases, and thus a stress is generated between bacteria that enables increased the production of bacteriocins. Also, the increase in the production can be given because encapsulation acts as a protection, as it separates the bacteriocins, released to the medium, from the spheres in which proteolytic enzymes are found.

Regarding bacteriocins in the aquaculture industry, several factors play a significant role in the potential use of this proteins as probiotics or bio preservatives bio. Researchs in aquatic organisms had shown that the production and diversity of bacteriocins in the aquatic environment is abundant (Bagenda *et al.*, 2008). As mentioned above, one of the biggest problems in the industry is the use of antibiotics to prevent diseases, which have several subsequent problems in farming centers as resistance is generated, thats why bacteriocins have many qualities that make them attractive as alternatives to antibiotics, because it had been shown that are nontoxic to eukaryotic cells and are recognized as safe for health (GRAS), being a safe alternative to traditional antimicrobials (Galvez *et al.*, 2008). It had also been shown that purified bacteriocins not affect the sensory qualities of seafood and that are stable to salinity concentrations. Furthermore, the reduced action spectrum of bacteriocins, compared to traditional antibiotics, limits the selective pressure for bacteria to develop resistance to these antimicrobial and that reduces the incidence of drug-resistant pathogens. Brillet *et al.* (2005) demonstrated that *Carnobacterium divergens* V41 produce a

bacteriocin that can be used as a biopreservant to inhibit the growth of *Listeria monocytogenes* in salmon.

It had also been suggested that the bacteriocins can be combined with some methods for antimicrobial treatment and preservation to produce synergistic effects, as the incorporation of bacteriocins in bioactive packaging (Calo-Mata *et al.*, 2007; Pilet and Leroi, 2011). Encapsulation of bacteriocins for biopreservation could reduce packaging costs due to the low amount and cost of the antibacterial necessary to join the gel (Galvez *et al.*, 2008).

Therefore, immobilized lactic acid bacteria producing a bacteriocin in combination with the appropriate method used in the aquaculture industry has a potential to increase the guarantee of freshness, ensuring the inhibition of microorganisms that cause spoilage.

Chapter 3: Materials and Methods

3.1. Microorganisms and media

The bacteriocingenic *P. acidilactici* LPS28 strain, isolated from the gastrointestinal tract of Atlantic salmon that was cultivated in the South of Chile, was used throughout the study. Several other strains were used as target bacteria to verify bacteriocin activity *i.e.*, *Lactobacillus curvatus* CTC 371, *Lactobacillus fermentum* strains LMG 6902, D13RR05S01, and 222, *Lactobacillus plantarum* strains 13201, 80, and LMG 6907, *Listeria innocua* strains ATCC 33090,CTC 1012, CTC 1014, LMG 11387^{T}, LMG 1354, LMG 13568, R25, and RZS, *Listeria ivanovii* LTH 3097, *P. acidilactici* strains LPS26 (another salmon isolate), ATCC 8042, *Pediococcus pentasaceus* LMG 13650 and LMG 13651, and *Staphylococcus aureus* strains E002, G111, i030, k134, L155, M104, SAU22, SAU5, and Z163. The two pediococcal salmon isolates, identified by partial 16S rRNA gene sequencing, and *L. innocua* ATCC 33090, obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), were maintained at -80°C in 25% glycerol/medium at the Laboratory of Bioprocessing at the Universidad de Concepción (Concepción, Chile), whereas the target strains were stored at the culture collection of the Research Group of Industrial Microbiology and Food Biotechnology of the Vrije Universiteit Brussel (Brussels, Belgium).

Prior to experimental use, the strains were propagated twice at 32°C in de Man, Rogosa and Sharpe (MRS) broth (Merck, Darmstadt, Germany); a first time for 24 h and a second time for 12 h. Solid media were prepared by adding 1.5 % (m/v) of agar (Merck).

The media used to investigate bacteriocin production by *P. acidilactici* LPS28 included: whey permeate (WP) [obtained from a local cheese producer and supplemented with peptone (Merck), yeast extract (Merck), sodium acetate (Merck), Tween 80 (Merck), dihydrogen potassium phosphate (Merck), magnesium sulphate (Oxoid, Basingstoke, England), and manganese sulphate (Oxoid) to match the concentrations of MRS medium], lactase-treated whey permeate (LWP), i.e. WP that was treated with lactase (Maxilact® L200, DSM Food Specialities, Netherlands), MRS medium (Merck), and modified MRS medium (mMRS), i.e. MRS broth composed from single ingredients (Merck) but without phosphates and Tween and with addition of 1 g l^{-1} of $CaCl_2$. All media were set at pH 6.0.

3.2. Characterization of an antilisterial bacteriocin produced by P. acidilactici LPS28 isolated from salmon.

3.2.1. Bacteriocin production assays as a function of pH and temperature

P. acidilactici LPS28 was inoculated (0.2%, v/v) in MRS broth and cultivated for 24 h. Prior to the inoculation, the pH of the medium was set to specific values in a range between pH 4 and 10, using 10 mol l^{-1} of NaOH or HCl. Different incubation temperatures were also investigated (20, 30 and 37°C). Bacteriocin activity against *P. acidilactici* LPS26 was measured in culture-free cell supernatants, obtained by centrifugation at 10.000 x g for 10 min (Haraeus Sepatech 2502, Labofuge Centrifuge, Osterode, Germany) and after pH adjustment to 7, using the well-diffusion method (Tagg and McGiven, 1971).

3.2.2. Characterization of the antimicrobial peptide

To characterize the antimicrobial spectrum of the bacteriocin produced by *P. acidilactici* LPS28, inhibitory activity of its 24 h culture supernatant was determined towards the set of indicator bacteria listed above. To investigate the bacteriocin's tolerance to enzymatic treatments, heat, or pH inactivation, *P. acidilactici* LPS26 and *L. innocua* ATCC 33090 were used as indicator organisms. Culture supernatants were treated with either proteinase K (30 U mg^{-1}, Merck), trypsin (40 U mg^{-1}, Merck), or α-amylase (130 U mg^{-1}, Merck) at a final concentration of 1 mg ml^{-1} in phosphate buffer 50 mM (pH 6.5). The supernatants were incubated with the enzymes at 37 ºC for 2 h, as described previously (Corsetti *et al.*, 2004). The effect of heat on bacteriocin activity of the supernatant was determined by treatment at 60 ºC, 80 ºC, and 100 ºC for 30 min, as well as by sterilization (121 ºC for 15 min). The stability of the antimicrobial activity of the supernatant of *P. acidilactici* LPS28 to different pH conditions was tested by varying the pH between 3 to 10, with 10 mol l^{-1} NaOH or HCl.

3.2.3 Purification of an antimicrobial peptide

In a first step, acid extracts were obtained from the culture supernatant by diluting it (1:1, v/v) in ultrapure water acidified with 0.1% trifluoroacetic acid (TFA; Merck) at a final pH of 3.9. After incubating in an ice bath for 30 min with gentle shaking, the extracts were centrifuged at 10.000 x g for 20 min at 4°C and stored at -70°C until use.

Then, solid phase extraction was carried out by loading the acid extracts onto Sep-Pack Vac C18 cartridges (Waters Corporation, Milford, MA) balanced in water acidified with 0.05% TFA. After washing with acidified water, three successive dilutions were performed with 5, 40 and 80% acetonitrile (Merck) in acidified water. The fractions obtained were lyophilized in a Speed-vac and reconstituted with 400 ul of ultrapure water (Mitta *et al.*, 1999). An inhibitory assay using ELISA plates was performed for the fractions (Bulet *et al.*, 1991; Concha *et al.*, 2004).

The active fraction (40% of ACN) was then purified via chromatography (Mitta *et al.* 2000a) with a Dionex Ultimate 3000 HPLC biocompatible series with diode array detector (Thermo Scientific, Sunnyvale, CA, USA). Aliquots of 250 µl of the selected fraction were subjected to reserved-phase HPLC on a Kromasil Reversa 300-5C18 column (Thermo Scientific,). Elution was performed with a linear gradient of 0 to 50% acetonitrile in acidified water with 0.05% TFA for 240 min at a flow rate of 4 ml min^{-1}. The corresponding maximum-absorbance (256 nm) fractions were collected into polypropylene tubes and dried in a Speed-vac SC100 (Savant, New York, USA) They were stored at -20 ° C until use, after which they were reconstituted in ultrapure water and their antimicrobial activity was measured. The active fractions were subjected once more to chromatography using a linear gradient of 5 to 40% ACN with TFA for 90 min and the same procedure described above was repeated.

3.2.4. Protein quantification

To quantify the protein of the fraction of 40% ACN, a colorimetric method with bicinchoninic acid was performed using The Pierce BCA Protein Assay Kit (Thermo Scientific), following the manufacturer's conditions and protocol.

3.2.5. LC-MS/MS analysis

Analysis by LC-MS/MS was performed using an UHPLC Ultimate 3000 Dionex system incorporating a reversed-phase Syncronis C18 (50 x 2.1 mm) column with a particle size of 1.7 microns, coupled to a triple-quadrupole TSQ VANTAGE Thermo Finnigan mass spectrometer (Thermo Scientific) as Mitta *et al.* (2000a). The sample was diluted to a concentration of 10 µg/ml and 1 µl was injected using a linear gradient of 0 to 50% acetonitrile for 30 min 0.1% v/v formic acid at a flow rate of 300 µl/min. The ESI ionization

conditions were 4000 volts cone voltage, a capillary temperature of 350 °C, a cone temperature of 380 °C, an auxiliary gas flow of 35 litre per min and a nebulizer gas flow of 60 litre per min.

3.2.6. Tris-Tricine SDS-PAGE

Tricine- SDS- PAGE gel was carried out according to Schägger (2006), including 6.0 M of urea to increase the resolution of small proteins. After electrophoresis, the gels were silver-stained.

3.2.7. Membrane permeabilization

Listeria innocua ATCC33090 was used as a target strain for a membrane permeabilization study. Listerial suspensions were washed with PBS and then brought to an optical density of 1. Then, 25 µl of this bacterial solution was added in each well of a 96 black wells microplate, with 10 µl of the active fraction for bacteriocin activity obtained from P.*acidialctici* LPS28 after solid phase extraction, 1 µl propidium iodide (1 mg ml^{-1}) and completed to a final volume of 50 µl using sterile PBS. It was incubated at room temperature in the dark for 1 h. Finally, the fluorescence intensity was read in a microplate reader (BioTek Instruments, Winooski, VT, U.S.A.), exciting at 530 nm and measuring propidium iodide emission at 620 nm. Melittin (Sigma-Aldrich, St. Louis, MO, U.S.A.), a membrane permeabilizing peptide, was used as positive control at a concentration of 10 µM., and as negative control, the bacterial solution was incubated with sterile PBS.

3.3. Growth and bacteriocin production by *Pediococcus acidilactici* LPS28 in fermentations as planktonic cells

3.3.1. Fermentations

To investigate the effect of the cultivation medium, temperature and pH on both growth and bacteriocin production of *P. acidilactici* LPS28, fermentations were carried out in 500 ml flasks equipped with the instrumentation for measurement and control of pH containing 200 ml of the medium. The flask containing the medium was heat-sterilized at 120°C for 15 min. The medium was inoculated with 0.2% (v/v) inoculum of *P. acidilactici*

LPS28. Moderate agitation was performed to ensure homogeneity of the broth. The pH was controlled by the addition of NaOH 10 M. Temperature stayed within 0.1 °C of the set point.

For the study of the effect of the culture media, four different media were used in the fermentations: whey permeate (WP) [obtained from a local cheese producer and supplemented with peptone (Merck), yeast extract (Merck), sodium acetate (Merck), Tween 80 (Merck), dihydrogen potassium phosphate (Merck), magnesium sulphate (Oxoid, Basingstoke, England), and manganese sulphate (Oxoid) to match the concentrations of MRS medium], lactase-treated whey permeate (LWP), i.e. WP that was treated with lactase (Maxilact® L200, DSM Food Specialities, Netherlands), MRS medium (Merck), and modified MRS medium (mMRS), i.e. MRS broth composed from single ingredients (Merck) but without phosphates and Tween and with addition of 1 g l^{-1} of $CaCl_2$. All the mediums were set up at pH 6.0.

To investigate the influence of temperature and pH, the fermentations were done in MRS broth. The studied temperatures were 25 °C, 28 °C, 32 °C, 37 °C at the optimum pH (6.0 ± 0.1) of growth and bacteriocin production. The effect of pH was studied at pH of 4.5, 6.0, 7.5, 8.5 at the optimum temperature of bacteriocin production (32 ± 0.1 °C). Growth experiments in MRS broth without pH regulation (initial pH after inoculation 6.0) were carried out at 32 °C. All experiments were done in duplicates.

Over a period of 36 h at constant time intervals of 3 h, samples were withdrawn aseptically to determine cell concentration by serial dilution and plating on MRS agar.

3.3.2. Bacteriocin activity assay

Bacteriocin activity was measured in the culture supernatant obtained after centrifugation at 10.000 x g for 10 min and after adjusted to pH 7, using the well-diffusion method (Tagg and McGiven, 1971). 50 µl of the supernatant were spotted in MRS agar plates containing the indicator strain *Pediococcus acidilactici* LPS26. The lawns were prepared by adding a 24 h culture, a 32 °C, of the indicator strain in MRS agar. After incubation at 32 °C for 18 h the arbitrary units (AU ml^{-1}) of the bacteriocin were determined as the reciprocal of the highest dilution showing inhibition of the indicator strain.

3.4. Growth and bacteriocin production by *Pediococcus acidilactici* LPS28 immobilized in calcium-alginate beads

3.4.1. Immobilization of Pedicoccus acidilactici LPS28 in alginate spheres

P. acidilactici LPS28 was cultured in 200 ml of MRS broth at 32°C for 18 h. Cells were harvested by centrifuged (10.000 x *g*, 10 min) (Haraeus Sepatech 2502, Labofuge Centrifuge, Osterode, Germany) and re-suspended in 25 ml of 0.85% (m/v) saline solution and mixed it with 100 ml of 2% (m/v) of alginate (Quimatic S.A., Santiago, Chile). The mixture was dripped in a solution of 0.5 M calcium chloride, maintained under magnetic stirring, using a peristaltic pump at 4 ml per min (Masterflex® L/S EW-77201-60, Cole Parmer, Illinois, USA). The formed calcium alginate beads were kept for 12 h at 4°C. Later, the spheres were washed with 0.85 % (m/v) of saline solution and kept at 4 °C until experimental use. The particle size distribution of the obtained spheres was determined using Microtec S3500 (Montgomeryville, US.A.) measuring 15 beads for each sample.

To study the effect of alginate concentration on produced bacteriocin titers, the same procedure was accomplished with 1.5, 1.75, 2.0, 2.25, 2.5 and 3% (m/v) of alginate.

3.4.2. Batch fermentations

The fermentations with immobilized *P. acidilactici* LPS28 were performed in Erlenmeyer flasks containing 200 ml of MRS broth at pH 6.0 and 32 °C for 36 h with continue stirring (Lab-Line instruments, mod. 685-1DC2M, Kelara, India). Eighty grams of spheres were inoculated in the flasks.

To study the effect of the medium on the bacteriocin production, four different media were used in the fermentations: whey permeate (WP) [obtained from a local cheese producer and supplemented with peptone (Merck), yeast extract (Merck), sodium acetate (Merck), Tween 80 (Merck), dihydrogen potassium phosphate (Merck), magnesium sulphate (Oxoid, Basingstoke, England), and manganese sulphate (Oxoid) to match the concentrations of MRS medium], lactase-treated whey permeate (LWP), i.e. WP that was treated with lactase (Maxilact® L200, DSM Food Specialities, Netherlands), MRS medium (Merck), and modified MRS medium (mMRS), i.e. MRS broth composed from single ingredients (Merck) but without phosphates and Tween and with addition of 1 g l^{-1} of $CaCl_2$.

Also, the reutilization of the spheres was studied by every 24 h filtered the beads under sterile conditions, washed with saline solution and replaced in fresh media for second, third and fourth cycle of fermentation.

3.4.3. Continuous fermentation with immobilized cells

Calcium alginate immobilized *Pediococcus acidilactici* LPS28 cells were packed into a previously sterilise, glass column reactor (5 cm i.d. x 20 cm) fitted with a water jacket. The total fermenting columns was 200 ml. The column was maintained at 32°C by continuously circulating water from a thermostatically controlled waterbath through the water jacket. The pH in the bioreactor was manteined at 6.0 and controlled using an automatic pH controller (Cole Palmer, Chicago, IL) and 15% ammonium hydroxide. Fresh LWP medium at different dilution rates (0.10-0.6 h^{-1}), was pumped through the column from the bottom upward using a peristaltic pump (Materflex). Samples were taken from the bioreactor at time intervals of 3 h for biomass (in the alginate beads and in the supernatant), and pediocin activity determinations as mentioned above. The volumetric biomass productivity in the beads and in the supernatant, and of pediocin was studied in function of the dilution rate.

3.4.4. Determination of cell growth and antimicrobial activity of immobilized *P. acidilactici* LPS28

Samples of both liquid media and spheres were taken every 3 h during the fermentation performances to determine the viable cell concentration (CFU) per ml or g and bacteriocin activity in arbitrary units (AU) per ml. To obtained cell concentration in the spheres, 3 g of spheres were dissolved in a citrate phosphate buffer (pH 7) for 4 h under continuous agitation, and in the liquid media it was measured directly by serial dilution on MRS agar and after incubation for 24 h at 32 °C. The antimicrobial activity was quantify against *P. acidilactici* LPS26 in the culture supernatant obtained by centrifugation (10000 x *g*, 10 min) and after pH adjustment at 7 using the well-diffusion method (Tagg and McGiven 1971).

To determine substrate consumption, glucose was measured by the GOD-PAP Method that is an enzymatic colorimetric test for glucose by the commercial kit GLUCOSE liquicolor (HUMAN Diagnostics, Wiesbaden, Germany)

3.4.5. Determination of the effective diffusion coefficient (D_e)

The effective diffusion coefficient of pediocin in calcium alginate beads was obtained by fitting the theoretical curve obtained by equation 3 to experimental data points of bacteriocin titers in the gel beads according to Willaert *et al.* , 1996)

$$M_A^t/M_A^\infty = 1 - [(6/\pi^2)[1/n^2 \sum_{n=1}^{\infty} \exp(-\frac{D_e n^2 \pi^2 t}{R^2})]] \qquad (3)$$

Where M_A^t is the amount of solute in the matrix at time t, M_A^∞, is he equilibrium amount, and R is the radius of the bead.

3.4.6. Scanning Electron Microscopy (SEM)

Spheres morphology was observed in the Scanning electron microscopy (SEM) ETEC model Autoscan-U 1 (ETEC Corporation Hayward, U.S.A.) belonging to the Electron Microscopy Laboratory of the University of Concepción. Protocols and methodology to prepare the samples before the scanning were reserved by the Laboratory.

3.4.7. Statistics

Parameter estimation was based on triplicate fermentation experiments, so that mean values and standard deviations were calculated for all cases. A Kruskal–Wallis one-way analysis of variance was used to test if model parameters displayed significant differences related to the media or alginate concentrations used. Where appropriate, a Mann-Whitney test was used to analyze specific pairs for significant differences.

3.5. Modelling growth and bacteriocin production by planktonic and alginate immobilized *Pediococcus acidilactici* LPS28

3.5.1. Model development

For the batch fermentations with planktonic cells, cell concentrations (X; in CFU per ml) over time (t; hours) were described with an equation based on the lag phase-accounting model of Baranyi and Roberts (1994):

$$dX/dt = \left(q/(q+1)\right)\mu_{max}\left(1 - X/X_{max}\right)X \quad (4)$$

where μ_{max} is the maximum specific growth rate (per hour), X_{max} is the maximum attainable cells concentration for a given set of conditions (in CFU per ml) and the dimensionless q is a capacity-type quantity expressing the proportion of the potential specific growth rate (which is determined by the actual environment) that is utilized by the cells.

For the experiments with alginate spheres, the cell concentrations of the immobilized cells (X_i; in CFU per g) were described in a similar manner. In addition, a number of cells detached spontaneously from the spheres into the medium as free, planktonic cells (X_f, in CFU per ml), which was described by a model taking into account a Jameson-like effect (Giménez and Dalgaard 2004):

$$dX_f/dt = \left(q/(q+1)\right)\mu_{max}\left(1 - X_f/X_{\max,f}\right)X_f\left(1 - X_i/X_{\max,i}\right) \quad (5)$$

with $X_{max,f}$ (in CFU per ml) and $X_{max,i}$ (in CFU per g) as the maximum attainable cells concentration of the free and immobilized cells, respectively, and where μ_{max} is the maximum specific growth rate (per hour) obtained for the experiments with planktonic cells [equation (4)]. This latter equation implies that all of the present bacteria must stop multiplying when the dominating microbiota (*in casu*, the immobilized cells in the alginate spheres) reaches maximum population density.

For the fermentations with planktonic cells of *P. acidilactici* LPS28, modelling of bacteriocin activity (B, in AU per ml) was based on a modification of a previously described approach for the description of bacteriocin activity by lactic acid bacteria (Leroy and De Vuyst 1999):

$$dB/dt = k_B\, dX/dt \quad (6)$$

with k_B as the specific bacteriocin production (in AU per CFU). The modification of the original equation consisted of a removal of an "apparent inactivation term", which was not relevant for the experiments in the present study.

For the fermentations with immobilized cells, a novel model for bacteriocin activity by immobilized cells needed to be developed to obtain satisfactory fitting of the data, since a shift in kinetics was encountered:

$$dB/dt = l_{B,i} X'_i \left(\frac{X_i}{X_{\max,i}}\right)^c \left(1 - \frac{B_i}{B_{\max,i}}\right) \quad (7)$$

where $l_{B,i}$ is the specific bacteriocin production rate (in AU per CFU per h) of the volume-converted concentration of immobilized cells X'_i (CFU per ml), $B_{max,i}$ is the plateau for bacteriocin activity levels (AU per ml), and c is a dimensionless shaping parameter. For comparison, bacteriocin activity was also modelled with equation (6), but based on the volume-converted cell growth of the immobilized cells and by using the k_B value obtained from the corresponding experiment with planktonic cells. Next, this k_B value was adjusted ($k_{B,ia}$, in AU per CFU) as to match the maximum bacteriocin levels obtained with the immobilized cells.

All equations were integrated with the Euler integration technique in Microsoft Excel. The parameters needed for the modelling were estimated by adjustment through the solver function until the best fit was obtained.

3.5.2. Statistics

Parameter estimation was based on triplicate fermentation experiments, so that mean values and standard deviations were calculated for all cases. A Kruskal–Wallis one-way analysis of variance was used to test if model parameters displayed significant differences related to the media or alginate concentrations used. Where appropriate, a Mann-Whitney test was used to analyse specific pairs for significant differences.

3.6. Use of *Pediococcus acidilactici* LPS28 to control listerial growth in smoked salmon.

3.6.1. Co-cultures of P. acidilactici LPS28 and LPS 26 and L. innocua ATCC 33090 in smoked salmon

Cells were harvested by centrifugation at 5.000 x *g* for 10 min (Haraeus Sepatech 2502, Labofuge Centrifuge, Osterode, Germany), washed two times with a 0.85-% (m/v) sterile saline solution and finally re-suspended in saline solution to obtain cell concentrations of approximately 1 x 10^9 colony-forming units (CFU) per ml for *P. acidilactici* strains, and 1 x 10^7 CFU/ml for *L. innocua* ATCC 33090.

Five thin slices of 25.0 g ± 1.0 g of smoked salmon were used for each set of experiments: (i) a slice inoculated with 10^7 CFU/cm^2 of *P. acidilactici* LPS28, (ii) a slice inoculated with 10^7 CFU/cm^2 of *P. acidilactici* LPS26, (iii) a slice inoculated with 10^5 CFU/cm^2 of *L. innocua* ATCC 33090, (iv) a slice inoculated with a co-culture of *P. acidilactici* LPS28 (10^7 CFU/cm^2) and *L. innocua* ATCC 33090 (10^5 CFU/cm^2), and (v) a slice inoculated with a co-culture of *P. acidilactici* LPS26 (10^7 CFU/cm^2) and *L. innocua* ATCC 33090 (10^5 CFU/cm^2). The slices were stored at both 4°C and 15°C. All experiment were done in duplicate.

To verify any potential inhibitory effects due to acidification by the pediococci against *L. innocua* ATCC 33090, the pH evolution was measured in all cases (HI 2221 Calibration Check pH/ORP Meter, Hanna instruments, Woonsocket, RI, U.S.A.).

3.6.2. Sampling and determination of cell growth and pediocin production in smoked salmon

Squares of 1 cm^2 were withdrawn aseptically and were homogenized for 15 min in 25 ml of a 0.85-% saline solution with a sonicator bath Elmasonic S30 (Elma Schmidbauer GmbH, Singen, Germany), to determine cell concentration in colony forming units (CFU) per cm^2 on MRS agar (indicative of pediococcal growth) and ALOA agar (Sigma, Buchs, Switzerland), a selective agar for *Listeria*. Bacteriocin activity was tested by a variation on the bacteriocin diffusion assay (Tagg and McGiven, 1971), whereby agar plates were prepared with an overlay of the indicator strain *L. innocua* ATCC 33090 and, before the agar

solidified, the salmon pieces were placed on the agar which was then incubated at 37°C for 15 h.

3.6.3. Statistical analyses

Statistical analyses were performed with STATGRAPHICS Centurion XVI (Statpoint Technologies, Virginia, U.S.A.). Mean values and standard deviations were determined. Significant differences among mono and co-cultures assays on smoked salmon were obtained using Mann-Whitney test with a p value ≤ 0.05 as a threshold for significance.

4.1. Characterization of an antilisterial bacteriocin produced by *P. acidilactici* LPS28 isolated from salmon.

4.1.1. Bacteriocin production

P.acidilactici LPS28 was able to produce an antimicrobial compound after a fermentation for 24 h in MRS broth at most of the pH values and temperatures studied (Table 1). However, at pH 10, *P.acidilactici* LPS28 could not grow and thus failed in producing the inhibitory substance. With respect to the effect of fermentation temperature, the antimicrobial compound was produced at temperatures between 20 and 37 °C.

Table 1: Effect of pH and temperature on bacteriocin production by P. acidilactici LPS28

Condition	Treatment	Activity
pH	4 - 9	+++
	10	-
Temperature (°C)	20	+++
	30	+++
	32	+++
	37	+++

4.1.2. Characterization of the inhibitory compound

P. acidilactici LPS28 inhibited all of the nine *Listeria* strains used in the present study (Table 2). Also, microorganisms closely related to the producer strain, like *P. acidilactici* LPS26, *P. acidilactici* ATCC 8042 and *P. pentasaceus* LMG 13561, as well as one *Lactobacillus plantarum* strain, were inhibited. The other lactic acid bacteria were not affected by the action of the antimicrobial compound produced by *P. acidilactici* LPS28. Moreover, no antistaphylococcal activity was found.

The antimicrobial compound retained its activity independent of the applied pH on the supernatant, only losing some activity at pH 10. In addition, resistance to heat was found, even at sterilization temperature. With relation to the enzymatic treatments, antimicrobial activity was lost in the presence of proteinase K, but not in the presence of trypsin and α-amylase (Table 3).

Table 2: Inhibition of different strains by *P. acidilactici* LPS28

Organism	Sensibility to bacteriocin
P. acidilactici LPS26	+++
P. acidilactici ATCC 8042	+++
P. pentasaceus LMG 13561	+++
P. pentasaceus LMG 13560	-
L. plantarum LMG 6907	+++
L. innocua 13568; 1012; 131187; 1354; 1014; R25; RZS; ATCC 33090	+++
L. ivanovii LTH 3097	+++
Staphylococcus spp (28)	-
L. fermentum (3)	-
L. curvatus CTC 371	-
L. plantarum (2)	-

*(+++) strong inhibition

Table 3: Effect of pH and temperature on bacteriocin activity

Condition	Treatment	Activity
pH	3 - 9	+++
	10	++
Temperature (°C)	60 (30 min)	+++
	80 (30 min)	+++
	100 (30 min)	+++
	121 (15 min)	+++
Proteolytic Enzymes	Trypsin	+++
	α - amylase	+++
	Lipase	-
	Proteinase K	-

*(++) intermediate (+++) strong inhibition

4.1.3. Protein purification

After having subjected the culture supernatant to acid and to solid phase extraction, three fractions from elution with acetonitrile (5%, 40% and 80%) were obtained and evaluated for antimicrobial activity assays using *L. innocua* ATCC 33090 as an indicator strain. Only the fraction of 40% ACN showed antimicrobial activity (data not shown).

This active fraction was then subjected to fractionation by reversed-phase HPLC and eluted with a gradient of 5-50% ACN (Figure 6). All peaks were collected, lyophilized and subsequently resuspended in 40 µl of ultrapure water. Next, inhibition assays for each fraction indicated activity for only one of the peaks (Figure 7 insert). The 40% ACN fraction presented a protein concentration of 247.87 µg/mL (data not shown).

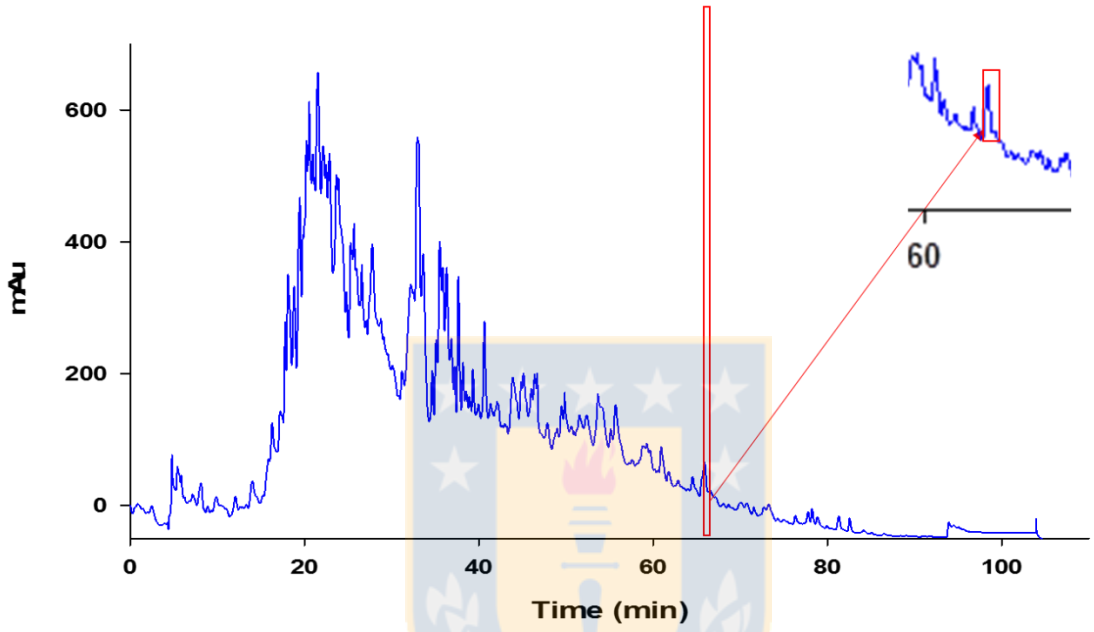

Figure 6: Purification of fraction of the 40% of ACN active against *L. innocua* ATCC 33090 by reversed-phase HPLC. The insert at the right displays the peak with antimicrobial activity.

A LC-MS of the peak that presented inhibitory activity against listeria was then performed. The molecular weight of the bacteriocin was determined by LC-MS/MS and estimated at 2247.36 Da (Figure 7).

Following Tris-Tricine SDS-PAGE, a doublet with a relative weight of approximately 7 kDa and 5 kDa was found, probably made up of aggregates, as well as a third, weaker band at about 3 kDa, probably consisting of the antimicrobial peptide (Figure 8).

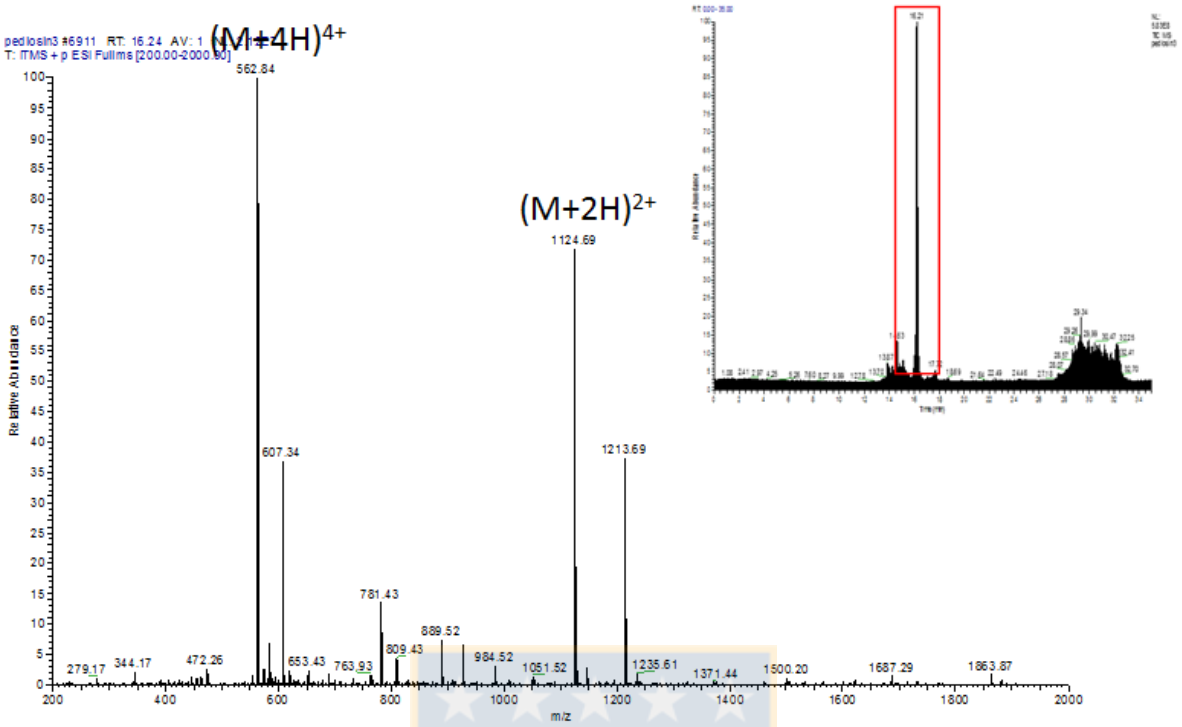

Figure 7: LC-MS (LTQ VelosPro) of the antilisterial compound.

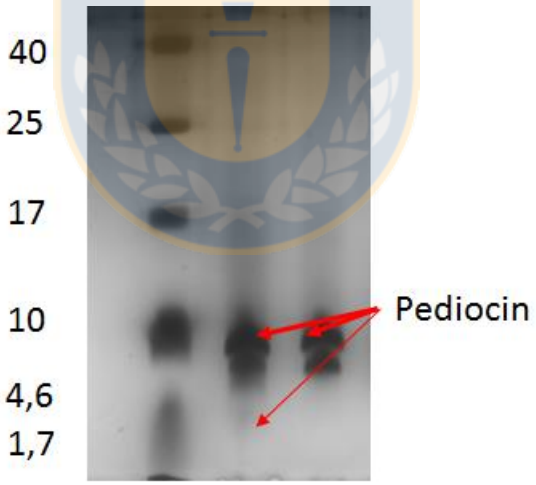

Figure 8: Tris-tricine SDS-PAGE at 16% with urea; gel stained with silver

4.1.4. Membrane permeabilizing activity.

The most commonly described antimicrobial mechanism for bacteriocins corresponds to the disruption of the cell membranes of target bacteria. Upon evaluation of the ability of P. *acidilactici* supernatant fractions of peptides to permeabilize membranes, a significant increase in fluorescence was observed, as compared to negative PBS control (Similarly to

Melittin's intensity, data not shown) (Figure 9). Relative fluoresce intensity is a parameter that indicates that the protein disrupted the membrane and the propidium iodide bind the DNA of the *Listeria*, showing fluoresce.

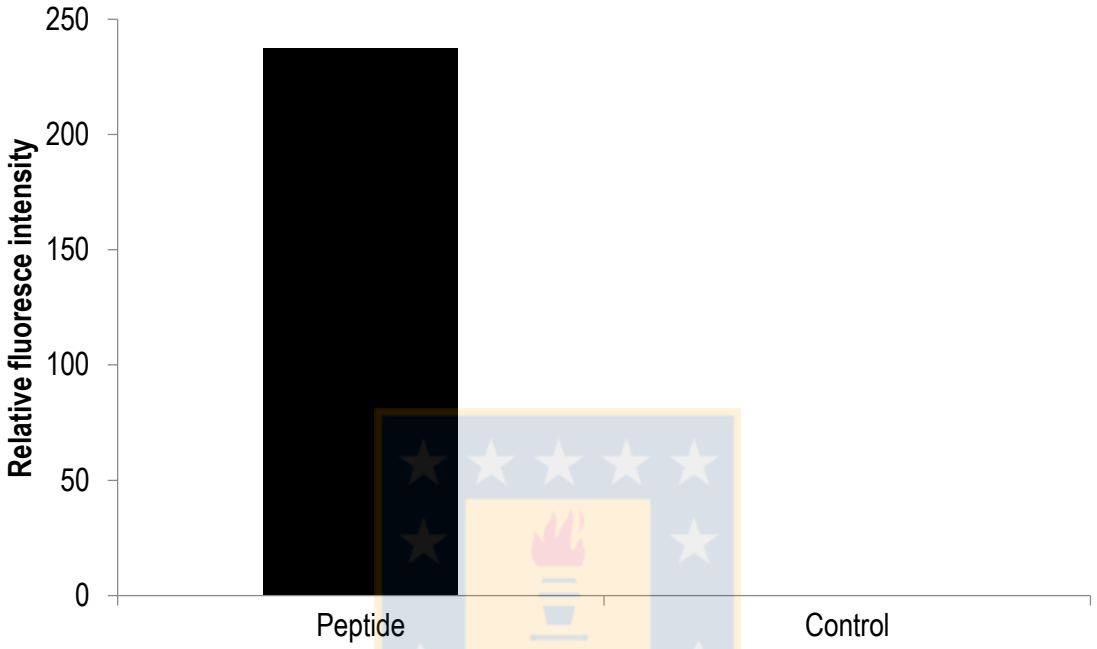

Figure 9: Membrane-permeabilising activity of the bacteriocin-containing extract from *P. acidilactici* LPS28, as compared to the control.

4.2. Growth and bacteriocin production by *Pediococcus acidilactici* LPS28 in fermentations as planktonic cells

4.2.1. Growth of P.acidilactici LPS28 and production of bacteriocins

Previous results indicated that the optimum conditions for growth of *P.acidilactici* LPS28 were at 32°C in MRS broth at initial pH 6. Bacteriocin production under that conditions at uncontrolled pH was studied (Figure 10). Bacteriocin production by *P. acidilactici* LPS28 followed primary metabolite kinetics, paralleling active cell growth. Detectable levels of bacteriocin appeared from the middle of the exponential phase on, *i.e.* after about 6 h of fermentation and when the cell population approached a concentration of about 7 Log CFU ml^{-1}. Maximum bacteriocin levels, of 2.6 x 10^5 AU ml^{-1}, were reached when the population reached its maximum level, *i.e.* between 8.5 and 9.0 Log CFU ml^{-1}, as bacteria entered the stationary phase after about 10 to 15 h. Subsequently, bacteriocin levels

remained constant. The pH on the culture broth decreased up to 4.3 showing lactic acid production.

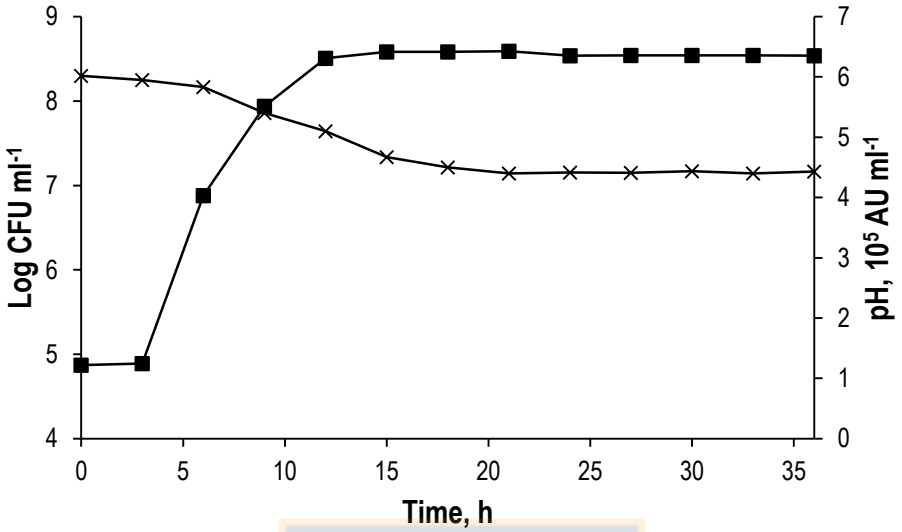

Figure 10: Growth of *Pediococcus acidilactici* LPS28 at uncontrolled pH at 32°C. (■) cell concentration, (□) bacteriocin activity, (×) pH.

4.2.2. Effect of fermentation medium on growth and production of bacteriocins.

The type of cultivation medium did have some effect on cell growth and clearly influenced the bacteriocin production (Figure 11 and 12). The fermentation broth had an effect on the growth of *Pediococcus acidilactici* LPS28, showing statically significant differences ($p = 0.03$) on the cell counts especially between WP and LWP. The growth profile between MRS and mMRS doesn´t showed statically significant differences ($p = 0.878$). The maximum cell concentration after reaching the stationary growth phase was obtained in LWP of 9,3 Log CFU ml^{-1} (Figure 11). Glucose consumption evolution was independent of the medium (Figure 11), showing similar profiles in the three cases studied ($p = 0,864$).

With respect to the bacteriocin production, a profound influence of the medium on the maximum obtained titres was found, with the best result obtained for growth of the producer strain in LWP. Statically significant differences ($p = 0.002$) were found between the titers obtained in different medium studied.

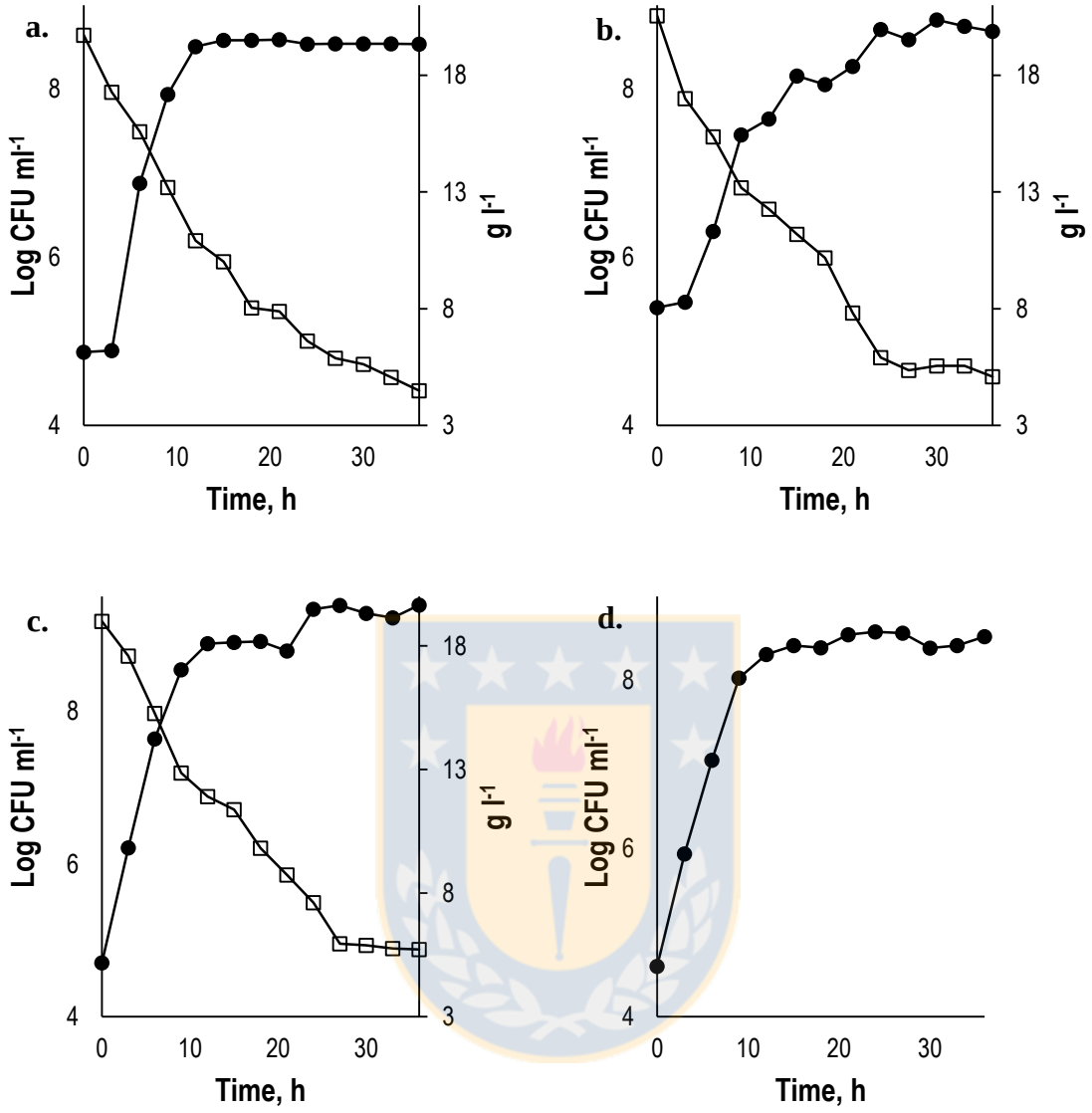

Figure 11: Effect of fermentation medium on the (●) growth of *P. acidilactici* LPS28 and (□) glucose consumption at 32°C at pH 6.0. (a) MRS broth, (b) mMRS, (c) LWP, (d) WP.

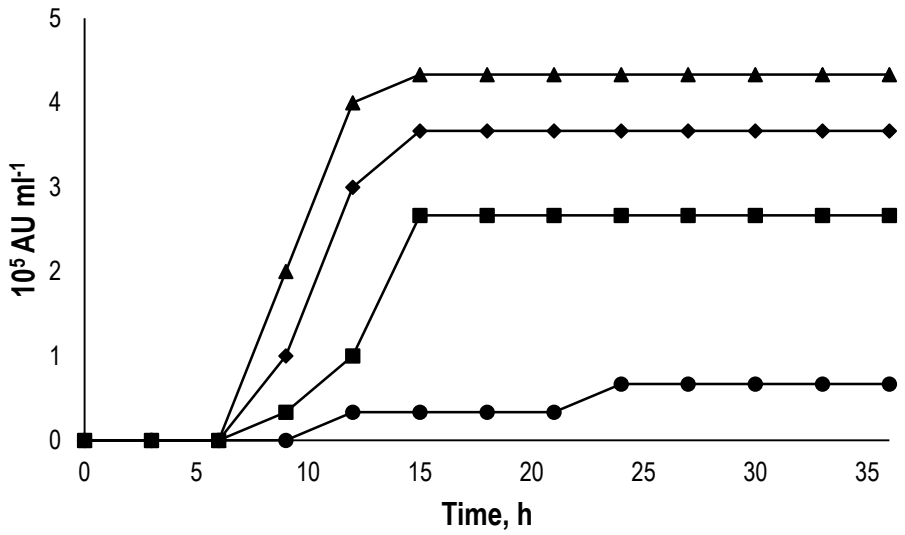

Figure 12: Effect of fermentation medium on bacteriocin production by *P. acidilactici* LPS28 at 32 °C at pH 6.0. (■) MRS broth, (♦) mMRS, (▲) LWP, (●) WP.

4.2.3. Effect of pH on growth and production of bacteriocins.

The pH had an effect on the final biomass of *P. acidilactici* LPS28 (Figure 13) and on bacteriocin production (Figure 14), and the results presented statically significant differences between the different cases studied either on the growth ($p < 0.001$) and the bacteriocin titers ($p = 0.039$). The highest final cell concentration was obtained at pH 6.0 near 8.5 Log CFU ml^{-1}. When the initial pH was 6.0 and it was uncontrolled during the fermentation, the final concentration was 8.2 Log CFU ml^{-1}; the evolution was lower than at constant pH ($p = 0.04$). At pH below 6.0, *P. acidilactici* LPS28 grew less and reached a final concentration 7.0 Log CFU ml^{-1}. At alkaline pH, final concentration can´t reached the 10^8 CFU ml^{-1}

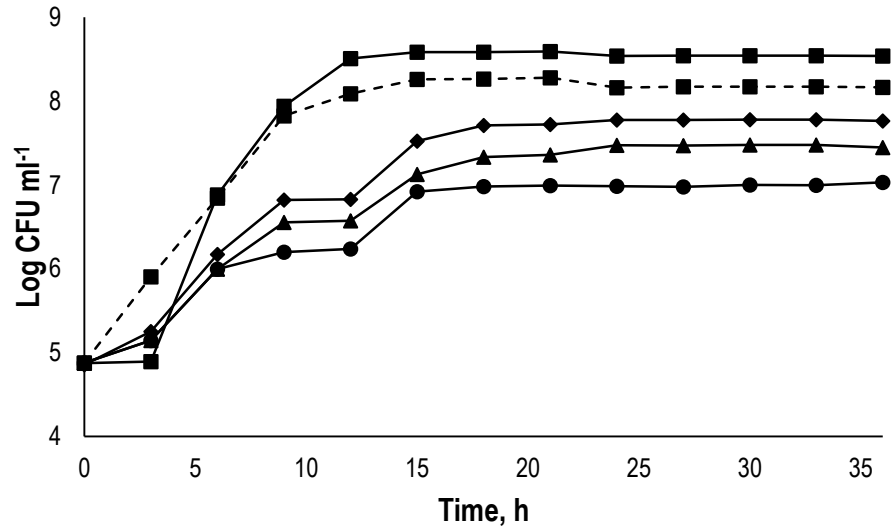

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Figure 13: Effect of different pH values on the growth of *P. acidilactici* LPS28 at 32 °C. (-■-) Uncontrolled pH, (■) pH 6.0, (♦) pH 7.0, (▲) pH 8.0, (●) pH 4.5.

Bacteriocin production was also influenced by the pH. Higher titers were obtained at pH 6.0 and uncontrolled pH, with no statically significant difference between the results ($p = 0.094$). Lowest titers were obtained with the other pH studied.

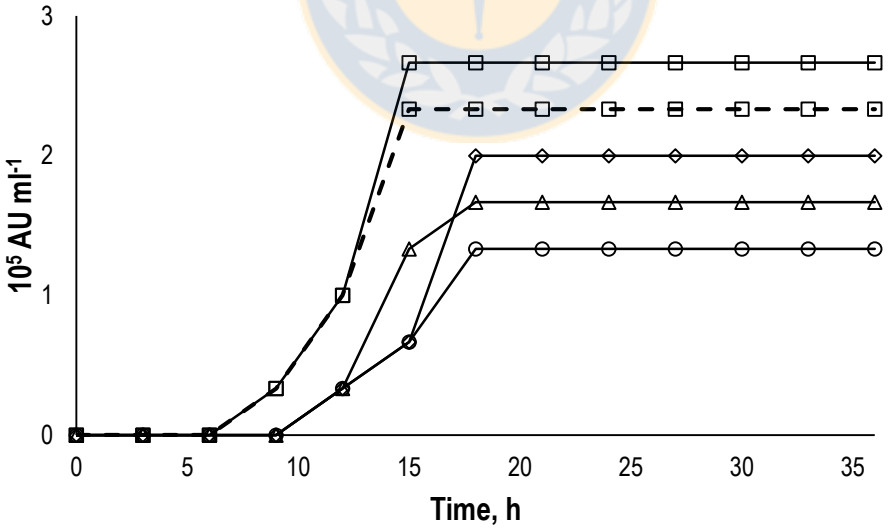

Figure 14: Effect of different pH values on bacteriocin production of *P. acidilactici* LPS28 at 32 °C. (-□-) Uncontrolled pH, (□) pH 6.0, (◊) pH 7.0, (Δ) pH 8.0, (○) pH 4.5.

4.2.4. Effect of temperature on growth and production of bacteriocins

The temperature had an effect on the final biomass of *P. acidilactici* LPS28 (Figure 15) and on bacteriocin production (Figure 16), and the results presented statically significant differences between the different cases studied on the growth ($p = 0.002$), but not on the bacteriocin titers ($p = 0.081$). The highest final cell concentration was obtained at 37 °C, and it was near 8.8 Log CFU ml^{-1}. When the temperature was 32 °C, the final concentration was 8.5 Log CFU ml^{-1}, results that were different statistically ($p = 0.031$) to 37 °C. At lower temperatures, *P. acidilactici* LPS28 grew less and reached a lower final biomass concentration. When the effect of temperature on bacteriocin production was studied, the highest final titer was obtained at 32 °C and 37 °C, and the lowest at 25 °C.

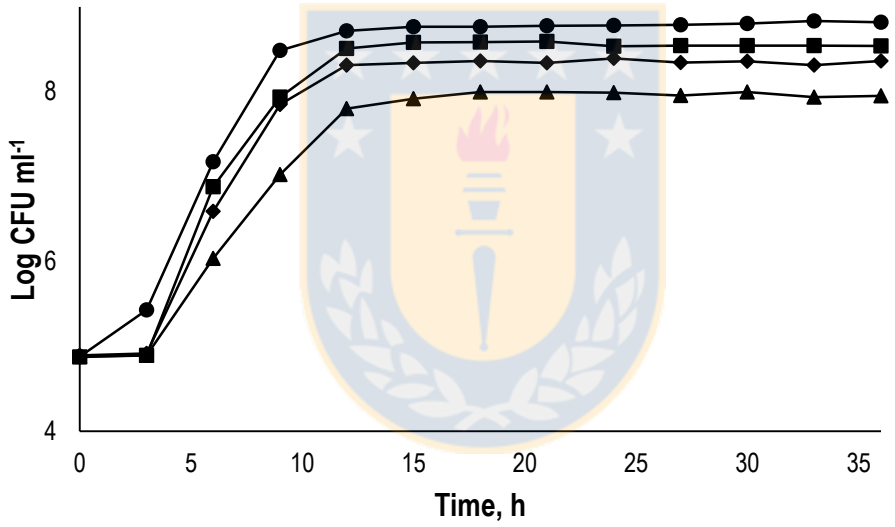

Figure 15: Effect of different temperatures values on the growth of *P. acidilactici* LPS28 at pH 6.0. (■) 32 °C, (♦) 28 °C, (▲) 25 °C, (●) 37 °C.

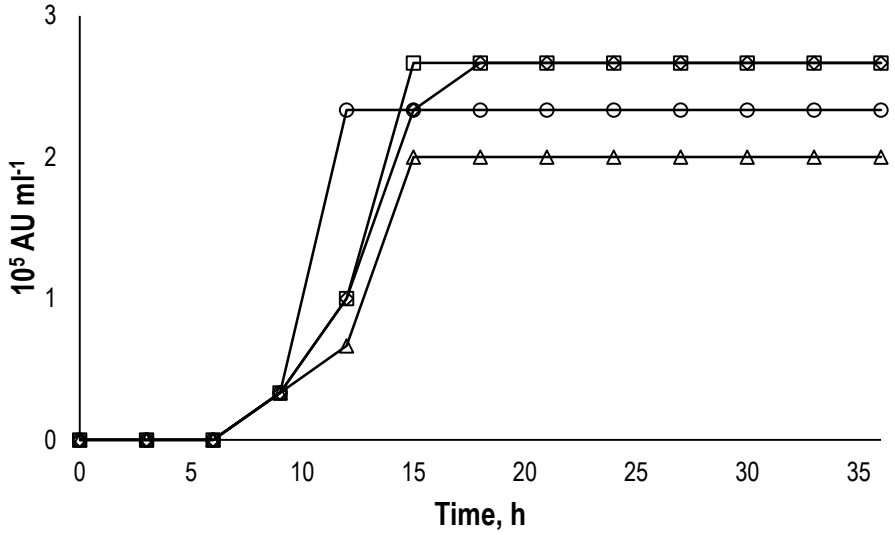

Figure 16: Effect of different temperatures values on bacteriocin production of *P. acidilactici* LPS28 at pH 6.0. (□) 32 °C, (◊) 28 °C, (Δ) 25 °C, (○) 37 °C.

4.3. Growth and bacteriocin production by *Pediococcus acidilactici* LPS28 immobilized in calcium-alginate beads

4.3.1. Growth and bacteriocin production by P. acidilactici LPS28 immobilized in alginate beads.

P. acidilactici LPS28 growth immobilized in alginate beads only 0.5 log CFU g^{-1}, maintaining its cells concentration during the 36 h of the fermentation. Cells concentration in the supernatant surrounded the spheres increase up to 10^7 at 9 h and maintained constant during the following hours (Figure 17). The latter indicates a release of bacteria from the beads mostly at the first hours of the fermentation. With respect to substrate consumption on the fermentation broth, it decreases from an initial concentration of 20 ± 1.3 to 4.5 ± 0.3 g l^{-1}. As glucose concentration decreases, bacteriocin production increases (Figure 18). Immobilized *P. acidilactici* LPS28 in fermentation batch in MRS produced after 36 h a final bacteriocin titer of 4.33 10^4 AU ml^{-1}. Its production starts after 6 h and reached the hightest titer at 18 h of fermentation. Also, the hightest bacteriocin titer was obtained when the glucose concentration started to be constant (Figure 18), so the production of antimicrobial peptide is asocciated to the substrate concentration in the media. Also, the particle size distribution obtained by Microtec S3500 showed a diameter average of 776,1 µm (data not shown).

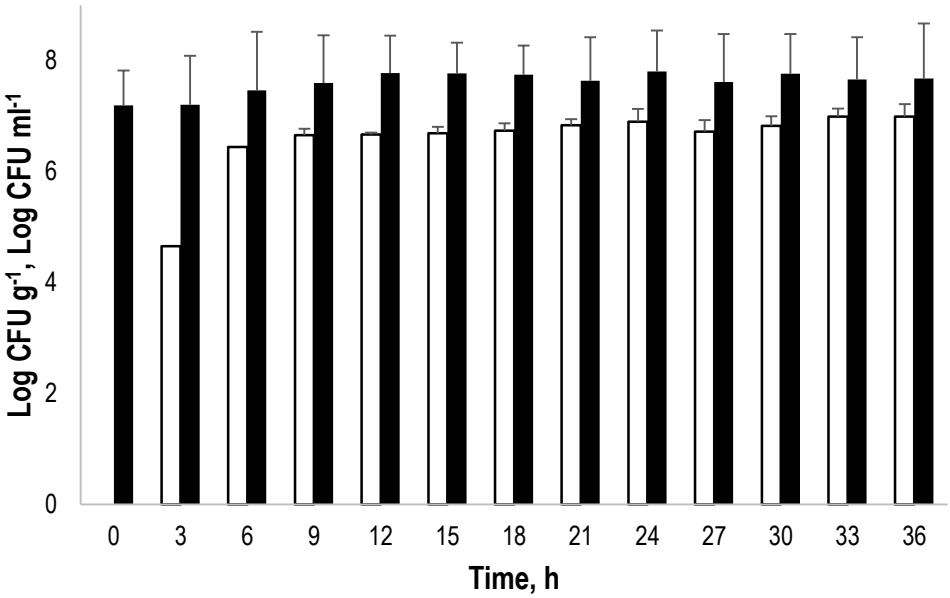

Figure 17. Counts of *P. acidilactici* LPS28 immobilized in calcium alginate beads (black bars) and in the media (white bars) during batch fermentation in MRS broth.

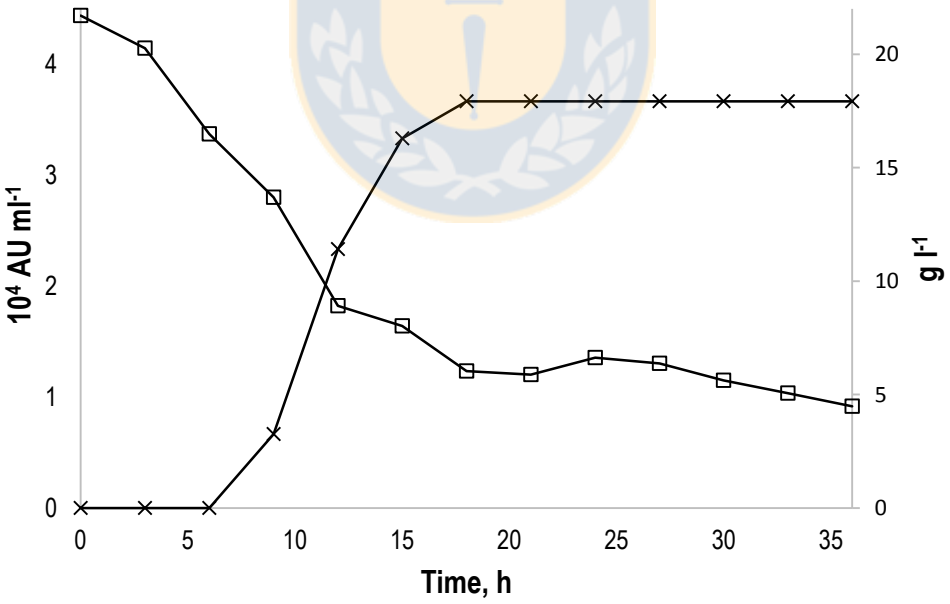

Figure 18. Glucose consumption (□) and bacteriocin production (×) by *P. acidilactici* LPS28 immobilized in calcium alginate beads during batch fermentation in MRS broth.

4.3.2. Effect of culture media on batch fermentations of immobilized P. acidilactici LPS28.

For experiments in MRS medium, mMRS, WP and LWP, cells of *P. acidilactici* LPS28 in the beads present a similar behavior ($p = 0.78$) reaching a final concentration near 8.2 ± 0.4 Log CFU g^{-1} in the four cases. Cells count in the surrounded media started to appear after 3 h of fermentation for MRS, mMRS and WP, and after 6 h for LWP. Cells concentration in the media is always lower that in the beads and in all the media studied reached a final count of 6.6 ± 0.4 Log CFU ml^{-1} (Figure 19).

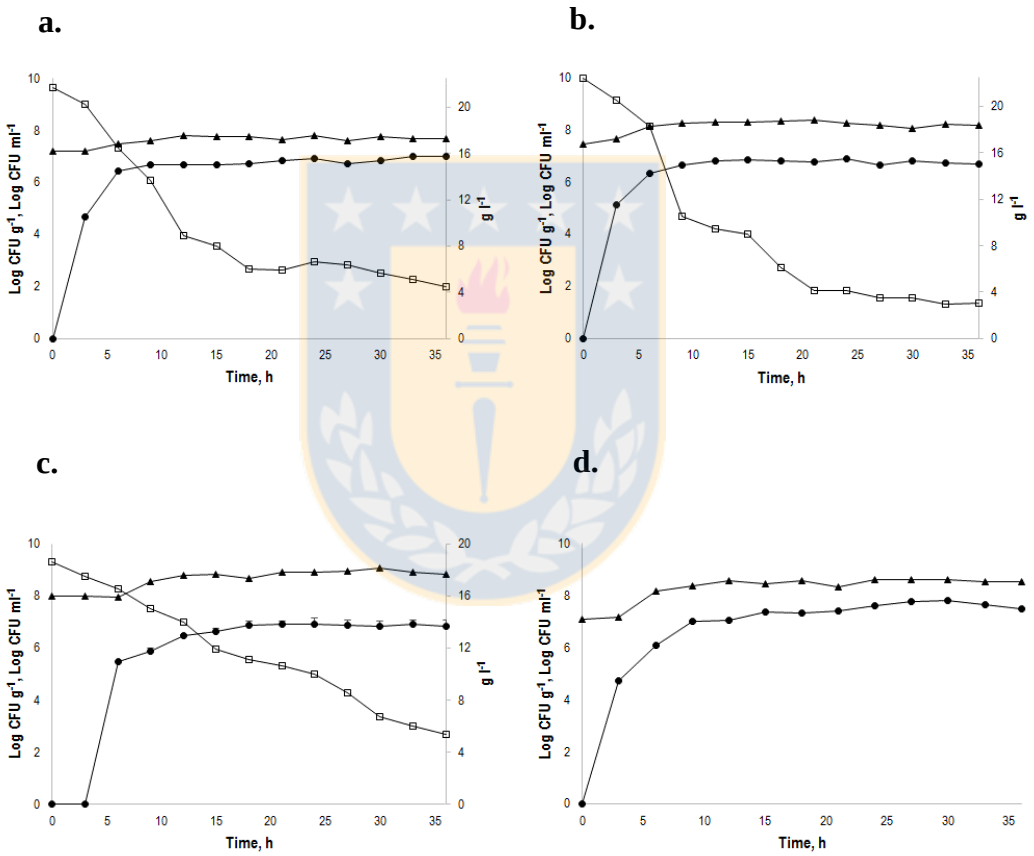

Figure 19. Counts of *P. acidilactici* LPS28 immobilized in calcium alginate beads (▲); in the surrounded media (●) and glucose consumption (□) during batch fermentation in MRS (a); mMRS (b); LWP (c) and WP (d) broth.

The glucose consumption present different profiles depending on the broth. For MRS and mMRS, glucose concentration decrease linearly in the first 12-15 h and then present a slight decrease reaching a constant plateau. For LWP, glucose consumption during the 36 h of batch fermentation of *P. acidilactici* LPS28 immobilized in calcium alginate beads present a linear decrease, remaining ¼ of the initial glucose concentration. In WP, no glucose was measured.

For bacteriocin production by immobilized *P. acidilactici* LPS28 in batch fermentations, different results were obtained depending on the medium ($p = 0.02$). In MRS medium, mMRS medium, and WP, the bacteriocin production started when the bacterial population in the alginate spheres already had reached the stationary phase. In the case of LWP, however, bacteriocin production was observed during the active growth phase of *P. acidilactici* LPS28 in the spheres. The highest bacteriocin titer was obtained in LWP medium being more than 10^4 AU ml^{-1} higher than the commercial MRS broth (Fig 20). Bacteriocin production was ascribed to the immobilized cells only, as the production by the free cells was negligible due to their low numbers.

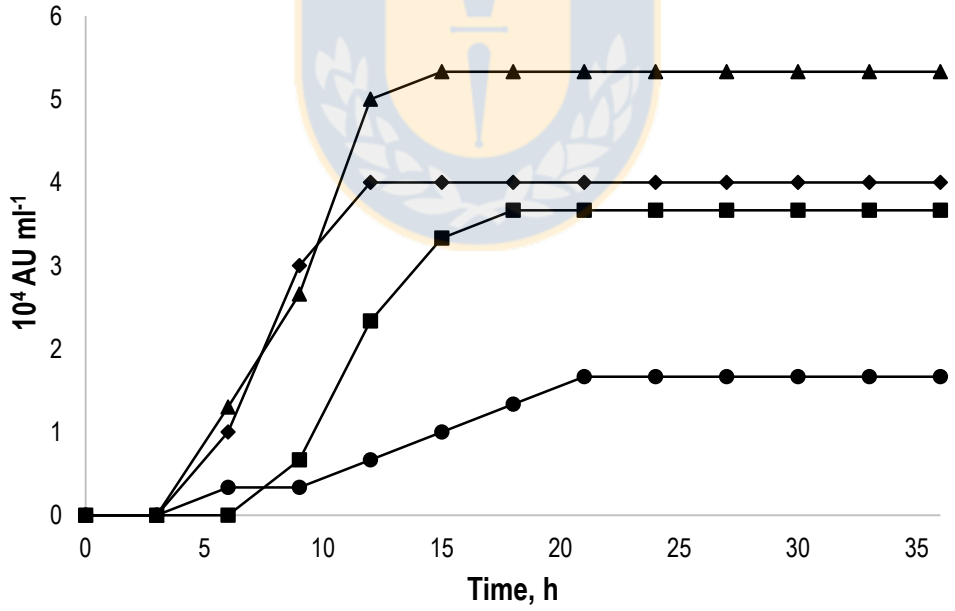

Figure 20. Bacteriocin production by *P. acidilactici* LPS28 immobilized in calcium alginate beads during batch fermentation in MRS (■), LWP (▲), WP (●), and WP (♦).

4.3.3. Spheres reutilization

Cells counts in alginate spheres with immobilized *P. acidilactici* LPS28 remained constant during the four 24 h-cycle fermentations demonstrating the stability of the beads (Figure 21). The released cell concentration at the end of each cycle was decreasing in comparison to the 10^7 CFU ml^{-1} obtained in the first cycle. At the fourth cycle, no bacteria was detectable in the media. Bacteriocin titers also decreased between each cycle, but as the complete media was replaced, only bacteriocin produced in that cycle was considered in the final concentration, indicating that the bacteria in the sphere are the responsible of the production since no bacteria was measured in the media and bacteriocin was produced.

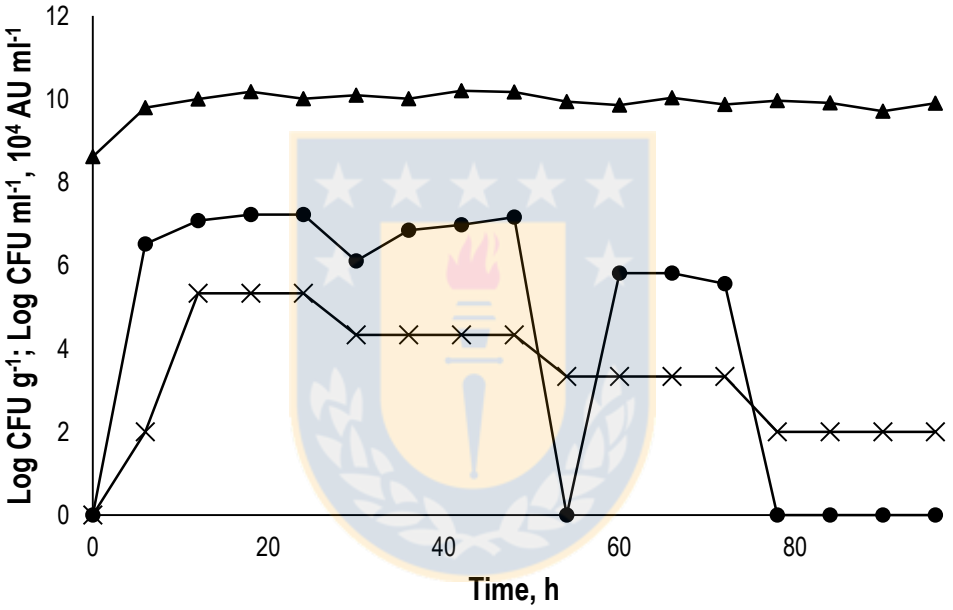

Figure 21. Counts of *P. acidilactici* LPS28 immobilized in calcium alginate beads (▲); in the surrounded media (●) and bacteriocin production (×) during repeated 24 h batch cycles fermentation in LWP broth.

4.3.4. Effect of alginate porcentage on bacteriocin production by immobilized P. acidilactici LPS28.

The percentage of alginate used in the immobilization process present a significant effect in the maximum bacteriocin concentration obtained in batch fermentations in LWP media with immobilized *P. acidilactici* LPS28. The highest bacteriocin titers were obtained at alginate percentages of 2, and the lowest at 1% (Table 4).

Table 4. Influence of the alginate concentration on B_{max} for *P. acidilactici* LPS28 immobilized in calcium alginate at 32°C in batch culture.

Alginate (%)	$B_{max} * 10^4$ (AU ml^{-1})
1.5	1
1.75	4,667
2	5,333
2.25	5,333
2.5	4
3	4

4.3.5. Determination of the effective diffusion coefficient (D_e)

The effective diffusion coefficient obtained by fitting experimental data of the bacteriocin titers in the beads with the theoretical curve obtained by eq. 3 showed in Figure 22, was of 2.5 x 10^{-5} cm^2/h.

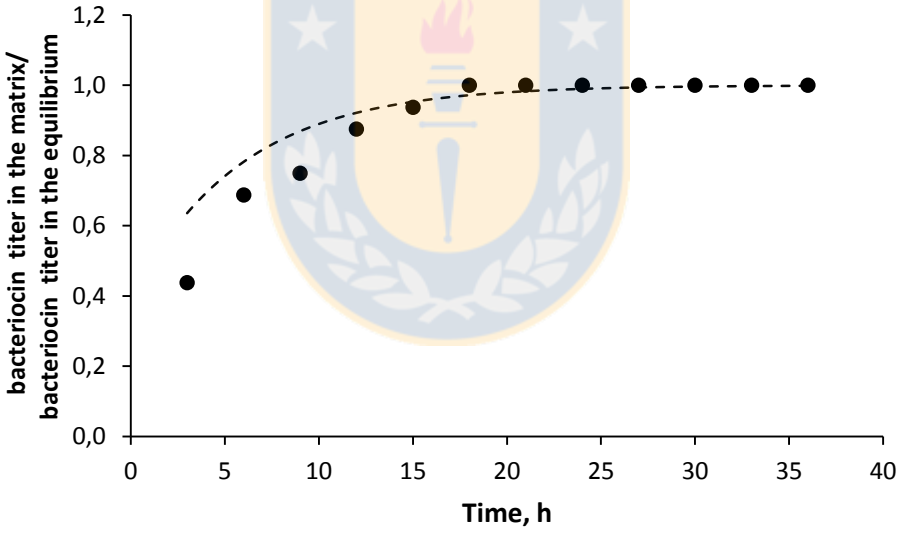

Figure 22: Bacteriocin production by *Pediococcus acidilactici* LPS28 in the calcium alginate beads. Experimental data (●), Theoretical data (- -)

4.3.6 Scanning Electron Microscopy

SEM shows that cell immobilization was achieved by entrapment of the cells in the void volume within the alginate matrix and attachment on the surface of it (Figure 23).

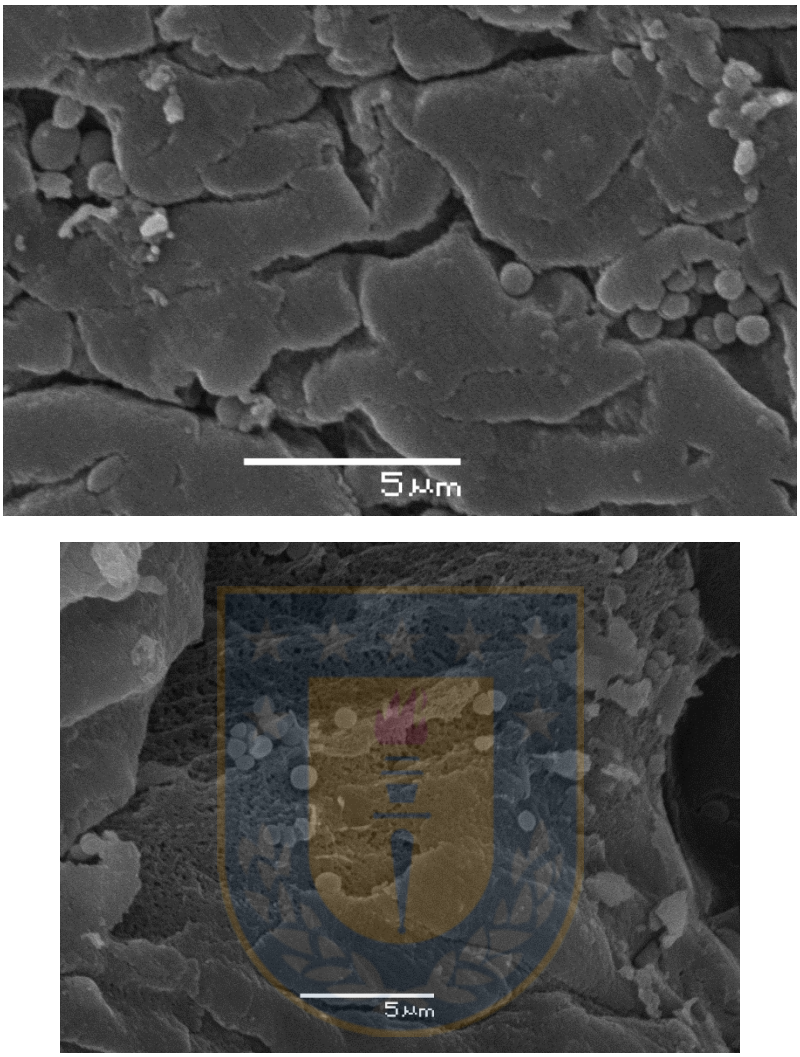

Figure 23: Micrograph from the scanning electron microscope, illustrating *P. acidilactici* LPS28 cell attachment onto calcium alginate matrix.

4.4. Modelling growth and bacteriocin production by planktonic and alginate immobilized *Pediococcus acidilactici* LPS28

4.4.1. Growth and production of bacteriocin by planktonic cells of *Pediococcus acidilactici* LPS28

During the batch fermentations with planktonic cells, bacteriocin producti4n by *P. acidilactici* LPS28 followed primary metabolite kinetics, paralleling active cell growth (Figure 24). The type of cultivation medium did have some effect on cell growth and clearly influenced the bacteriocin production (Figure 24, Table 5). Maximum population levels X_{max}

were in the range of 8.6 to 8.9 Log CFU ml^{-1} for all media, whereas some differences in maximum specific growth rates μ_{max} were found. Yet, a Kruskal-Wallis test indicated that this effect was not a significant one ($p = 0.06$). With respect to the bacteriocin production, a profound influence of the medium on the maximum obtained titres was found, with the best result obtained for growth of the producer strain in LWP. This disparity in maximum titres was explained by significant differences in the values of the specific bacteriocin production parameter k_B between the different media ($p = 0.02$). In comparison with LWP, WP yielded a lower maximum bacteriocin production, reflected in a significantly decreased k_B value ($p = 0.02$).

4.4.2. Growth and production of bacteriocin by immobilized cells of *Pediococcus acidilactici* LPS28

Following the introduction of the alginate spheres in the fermentation medium, a small amount of cells of the immobilized population of *P. acidilactici* LPS28 must have detached from the spheres during the first couple of hours and started to grow as planktonic cells in the liquid phase (Figure 25). The growth behaviour of these free cells was modelled based on the maximum specific growth rates obtained from the fermentations with planktonic cells only (Table 5). As the maximum population levels of these free cells were more than two Log CFU ml^{-1} below the levels obtained in the fermentations with planktonic cells only, entry into the stationary phase was related to the leading population of immobilized cells via an approach based on the Jameson effect. Overall, immobilized cells grew somewhat slower and reached lower maximum population levels (Table 6) than in the experiments with planktonic cells only (Table 4). Although growth rates levels of the immobilized cells may have been affected by the medium type or the alginate concentration, this was not considered significant ($p = 0.78$ and 0.22, respectively) (Tables 6 and 7). Yet, the maximum population was reached in WP which was the medium where the lowest bacteriocin production was obtained.

For experiments in MRS medium, mMRS medium, and WP, the bacteriocin production started, or at least became detectable, when the bacterial population in the alginate spheres already had reached the stationary phase (Figure 25). In the case of LWP, however, bacteriocin production was detectable during the active growth phase. Generally, bacteriocin

production was ascribed to the immobilized cells only, as the production by the free cells was negligible due to their low numbers. To illustrate this point, a simulation of bacteriocin production by the free cells was carried out, based on their growth curve and the k_B values obtained from the fermentations with planktonic cells (data not shown). This simulation indicated that production levels by the free cells never exceeded 240 AU ml^{-1}, being less than 0.5% of the production generated by the immobilized cells that were present in the alginate spheres.

Table 5. Influence of the medium on the model parameters and the maximum bacteriocin titers of *P. acidilactici* LPS28 during fermentations with planktonic cells at 32°C

Medium	μ_{max} (h^{-1})	X_{max} (CFU ml^{-1})	k_B (10^{-5} AU CFU^{-1})	Maximum bacteriocin titer (10^4 AU ml^{-1})[a]
WP	1.03 ± 0.01	8.64 ± 0.02	1.56 ± 0.08	0.7
LWP	1.29 ± 0.24	8.87 ± 0.56	9.51 ± 0.11	5.3
MRS	1.53 ± 0.05	8.72 ± 0.01	4.66 ± 0.03	2.7
mMRS	0.95 ± 0.35	8.66 ± 0.05	7.84 ± 0.60	3.7

[a] Results were identical for all three repetitions

When comparing different media, bacteriocin activity differed ($p = 0.02$) and was optimal in LWP (Table 5). The maximum bacteriocin production in LWP was obtained with 2.00 and 2.25 % of alginate, as also reflected by the corresponding l_{Bi} values. With the exception of the fermentation in LWP with 1.50 % of alginate, the experimental bacteriocin production was always considerably lower than the one predicted with equation (3), when using the cell growth of the immobilized cells and the k_B value derived from the planktonic cell experiments (Figure 25). When using the same equation but adjusting the k_B value to

$k_{B,ia}$ as to match the final bacteriocin concentration, up to six times higher specific production values were found (Tables 1-3). In addition, the simulated bacteriocin evolution paralleled the experimental data but was somewhat shifted to the left for most fermentations, suggesting diffusion limitations (Figure 25).

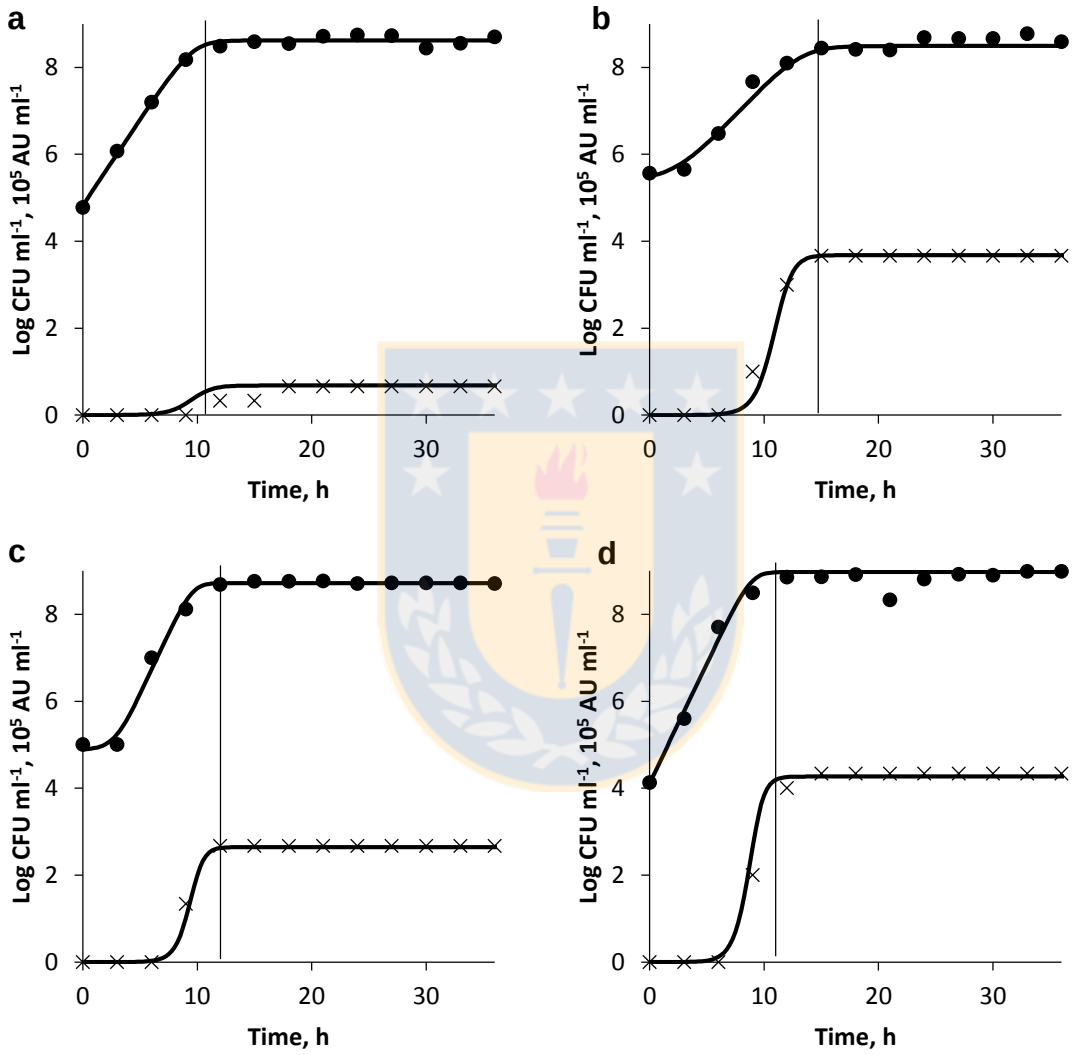

Figure 24: Modelling of biomass (●) and bacteriocin production (X) of *P. acidilactici* LPS28 cultivated as free cells at 32ºC in WP (a), LWP (b), MRS medium (c), and mMRS medium (d). Representative examples out of three repetitions are shown. Symbols represent experimental data; lines are according to the model. Vertical lines indicate the start of the stationary phase.

Table 6. Influence of the medium on the model parameters and the maximum bacteriocin titers of *P. acidilactici* LPS28 during fermentations with immobilized cells in calcium alginate (2%) at 32°C

Medium	$\mu_{max,i}$ (h^{-1})	$X_{max,i}$ (CFU ml^{-1})	$l_{B,i}$ (10^{-5} AU CFU^{-1} h^{-1})	$k_{B,ia}$ (10^{-5} AU CFU^{-1})	Maximum bacteriocin titer (10^4 AU ml^{-1})[a]
WP	0.65 ± 0.03	8.54 ± 0.07	1.68 ± 0.29	3.81± 0.17	1.7
LWP	0.71 ± 0.49	8.00 ± 0.18	52.3 ± 3.0	57.2 ± 4.43	5.3
MRS	1.12 ± 0.59	7.90 ± 0.59	10.1 ± 1.0	27.5 ± 4.44	3.7
mMRS	1.14 ± 0.63	8.23 ± 0.01	11.5 ± 2.12	27.9 ± 1.15	4.0

[a] Results were identical for all three repetitions

Table 7: Influence of alginate concentration on the model parameters and the maximum bacteriocin titers of *P. acidilactici* LPS28 during fermentations with immobilized cells in LWP at 32°C

Alginate (%)	$\mu_{max,i}$ (h^{-1})	$X_{max,i}$ (CFU ml^{-1})	$l_{B,i}$ (10^{-5} AU $CFU^{-1}h^{-1}$)	$k_{B,ia}$ (10^{-5} AU $CFU^{-1}h^{-1}$)	Maximum bacteriocin titer (10^5 AU ml^{-1})[a]
1.50	0.88 ± 0.08	8.46 ± 0.02	4.00 ± 0.09	3.66 ± 0.17	1.0
1.75	0.98 ± 0.02	8.51 ± 0.07	11.6 ± 0.46	16.9 ± 3.90	4.7
2.00	0.71 ± 0.49	8.00 ± 0.18	52.3 ± 3.00	57.2 ± 4.43	5.3
2.25	0.85 ± 0.66	8.40 ± 0.21	47.0 ± 2.01	26.8 ± 1.19	5.3
2.50	1.19 ± 0.36	8.12 ± 0.07	22.5 ± 0.36	33.1 ± 5.97	4.0
3.00	1.41 ± 0.18	8.23 ± 0.01	17.0 ± 0.22	24.5 ± 7.70	4.0

[a] Results were identical for all three repetitions

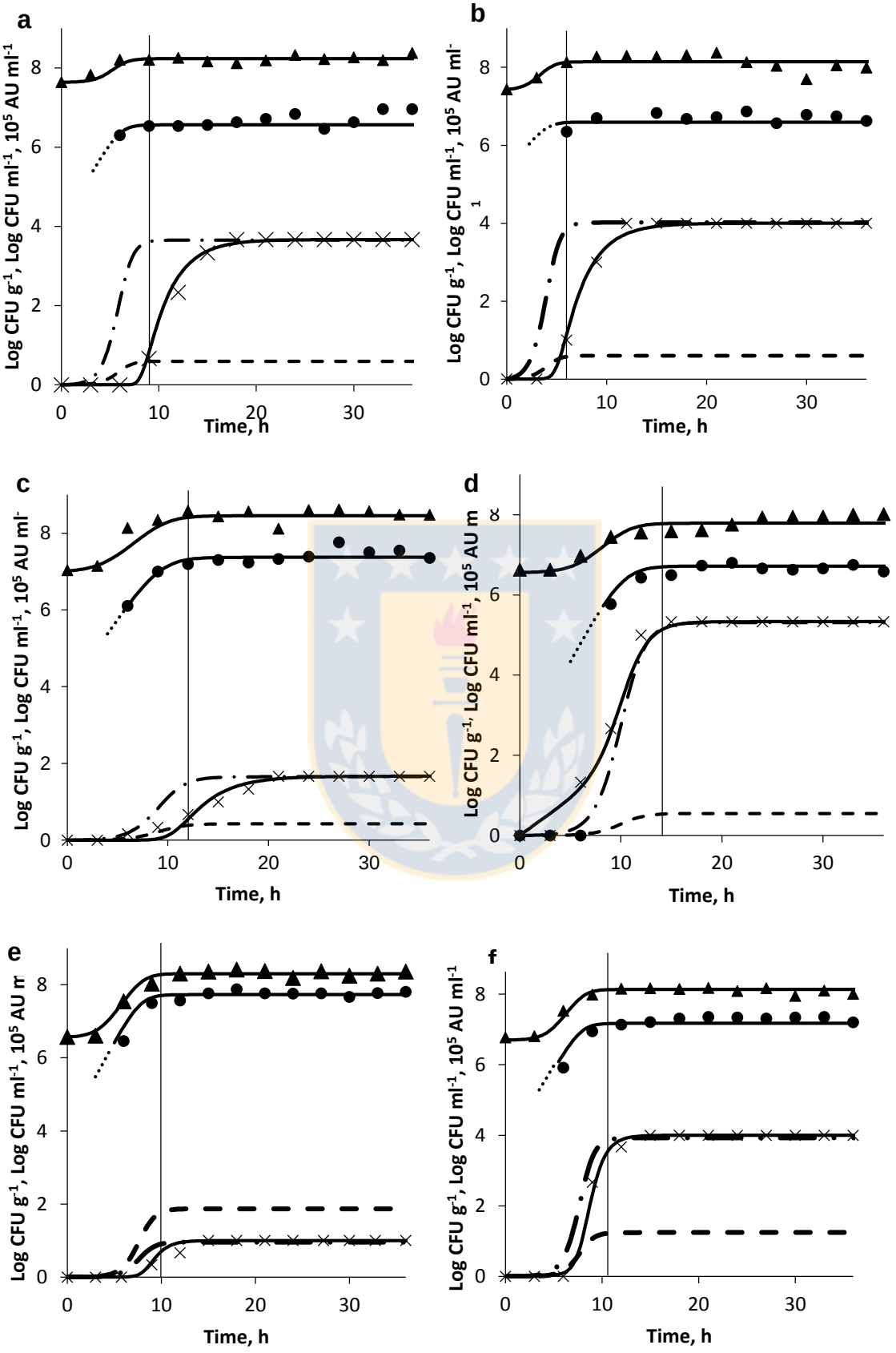
a
b
c
d
e
f
Log CFU g-1, Log CFU ml-1, 10^5 AU ml-1
Time, h
0
2
4
6
8
0
10
20
30

Figure 25: Modelling of biomass in the spheres (▲), in the supernatant (●), and bacteriocin production (x) of immobilized *P. acidilactici* LPS28 cultivated at 32°C in calcium alginate beads in MRS medium (a), mMRS medium (b), WP (c), and LWP (d) with 2.0% of alginate, as well as in LWP with 1.5% (e) and 3.0% of alginate (f). Representative examples out of three repetitions are shown. Symbols represent experimental data; lines are according to the model. Dashed lines indicate a simulation of bacteriocin production with equation [3], based on growth of the immobilized cells using the corresponding k_B value of the free cells (- -) or with an adjusted specific production ($k_{B,ia}$) to match the final experimental bacteriocin level (- . -). Vertical lines indicate the start of the stationary phase.

4.5. Continuous production of bacteriocin using calcium alginate-immobilized *Pediococcus acidilactici* LPS28.

4.5.1. Dilution rate

Continuous production of pediocin was achieved by using Ca-alginate-immobilized cells of *P. acidilactici* LPS28. The variations in the volumetric productivity with the dilution rate is presented in Figure 26. A low and constant increase is observed until dilution rate of 0,25 h^{-1} followed by a decline. At this point, the volumetric productivity was superior to 9,5 x 10^5 AU ml^{-1} h^{-1}.

The total biomass production rate, in both the supernatant and in the calcium alginate gel beads, increased with the dilution rate (Figure 27). In the supernatant, the biomass volumetric productivity reached up to 4,5 x 10^9 CFU ml^{-1} h^{-1} at a D of 0,6 h^{-1}. The gel beads, the biomass volumetric productivity increased with D up to a maximum above 0,45 h^{-1}.

Biomass within the gel beads was at all the dilution rates about 100 times higher than the average biomass concentration in the free-cells phase.

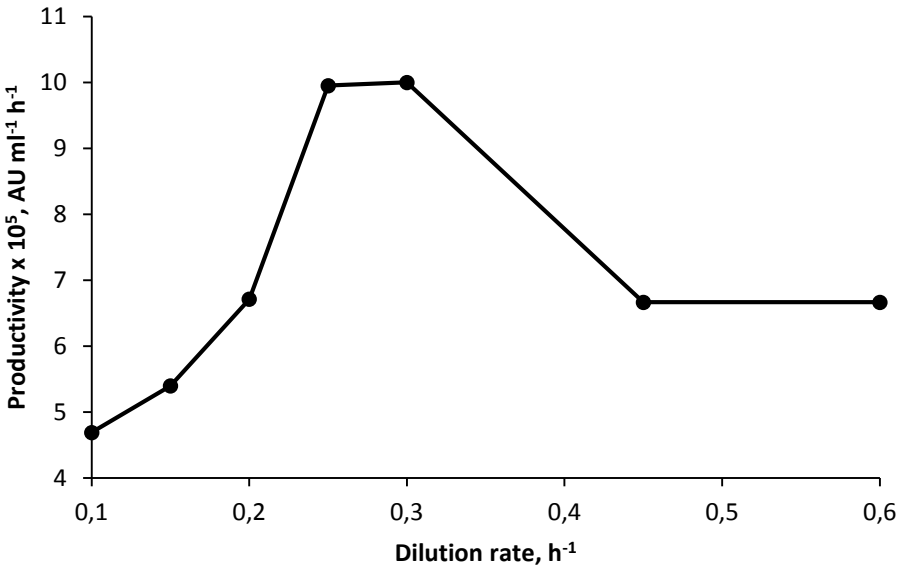

Figure 26. Variations of the bacteriocin volumetric productivity with the dilution rate for continuous fermentation with Ca-alginate immobilized *Pediococcus acidilactici* LPS28 cell filled in the packed bed bioreactor.

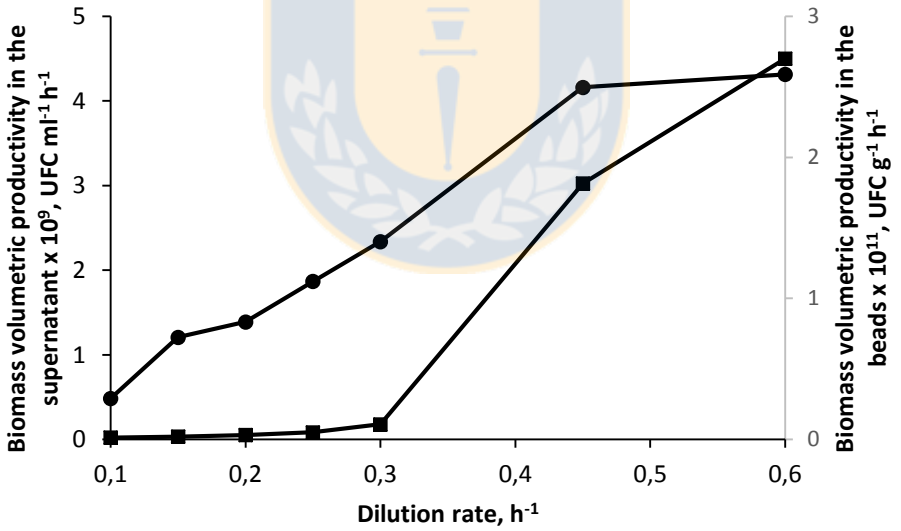

Figure 27. Variations of the biomass volumetric productivity in the supernantant (■) and in the gel beads (●) with the dilution rate for continuous fermentation with Ca-alginate immobilized *Pediococcus acidilactici* LPS28 cell filled in the packed bed bioreactor.

4.6. Use of *Pediococcus acidilactici* LPS28 to control listerial growth in smoked salmon.

4.6.1. Growth of P. acidilactici LPS28 as a monoculture and co-culture in smoked salmon

When adding a monoculture of *P. acidilactici* LPS28 to smoked salmon and incubating the product at 15°C for 24 h, the cell concentration as measured on MRS agar increased slightly (about 0.5 log CFU cm^{-2}) over time during the first day (Figure 28). When *P. acidilactici* LPS28 was added as a co-culture together with *L. innocua* ATCC 33090, similar cell concentrations were obtained as when the strain grew alone ($p = 0.2307$). In both cases, the pH decreased from pH 6.5 ± 0.1 to pH 4.5 ± 0.1, albeit somewhat faster for the monoculture experiment. For the experiments with *P. acidilactici* LPS26, the results were very comparable (Figure 28).

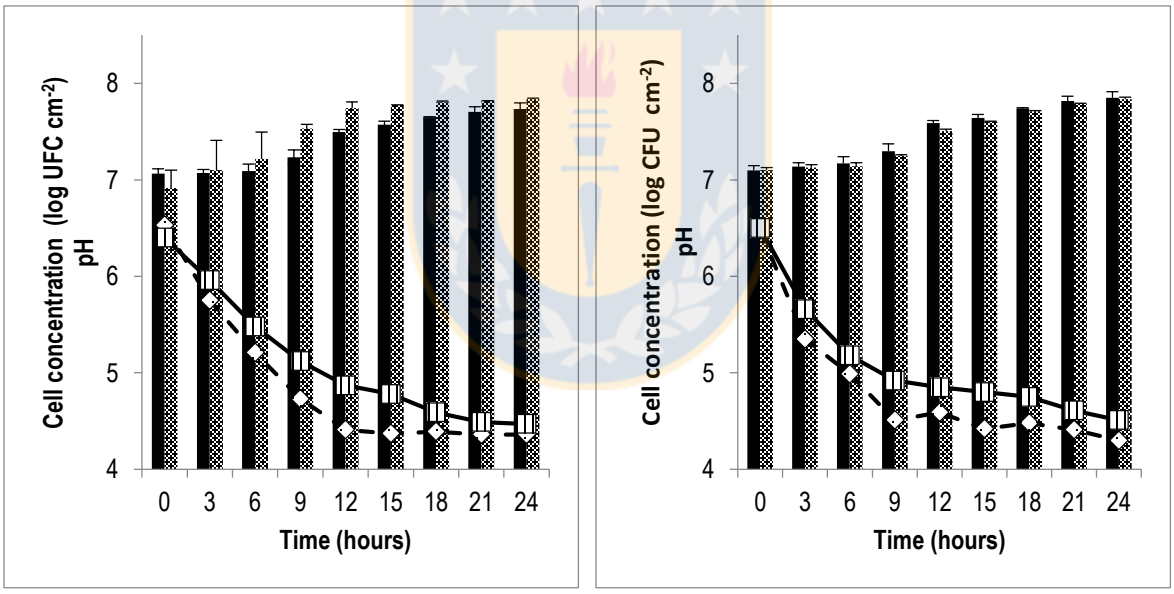

Figure 28: Evolution of the cell concentrations of *P. acidilactici* LPS28 (left) and LPS26 (right) as measured on MRS agar for the monoculture (■) and co-culture with *L. innocua* ATCC 33090 (▒) in smoked salmon during storage at 15°C for 24 h; pH in the monoculture (◇) and in the co-culture (⊟).

4.6.2. Growth of L. innocua ATCC 33090 as a monoculture and co-culture in smoked salmon

The evolution of the cell counts of *L. innocua* ATCC 33090 was clearly affected by the bacteriocin-producing *P. acidilactici* strain LPS28 but not by the non-bacteriocinogenic LPS26 strain. In the absence of added pediococci, the listerial cells reached a concentration of 8 log CFU cm^{-2}, whereas the pH of the experiment was constant at 7.0 ± 0.3 and the counts on MRS increased from 5.24 to 8.14 log CFU cm^{-2}. When the slices of salmon were inoculated with a co-culture of *L. innocua* ATCC 33090 and either *Pediococcus* strain, the pH decreased down to 4.47 after 24 h. In the experiment with *P. acidilactici* LPS26, the final *Listeria* concentration resembled the one obtained in the monoculture without added pediococci, but when *L. innocua* ATCC 33090 was grown with *P. acidilactici* LPS28, the cell concentration decreased with 2 log CFU cm^{-2} (Figure 29).

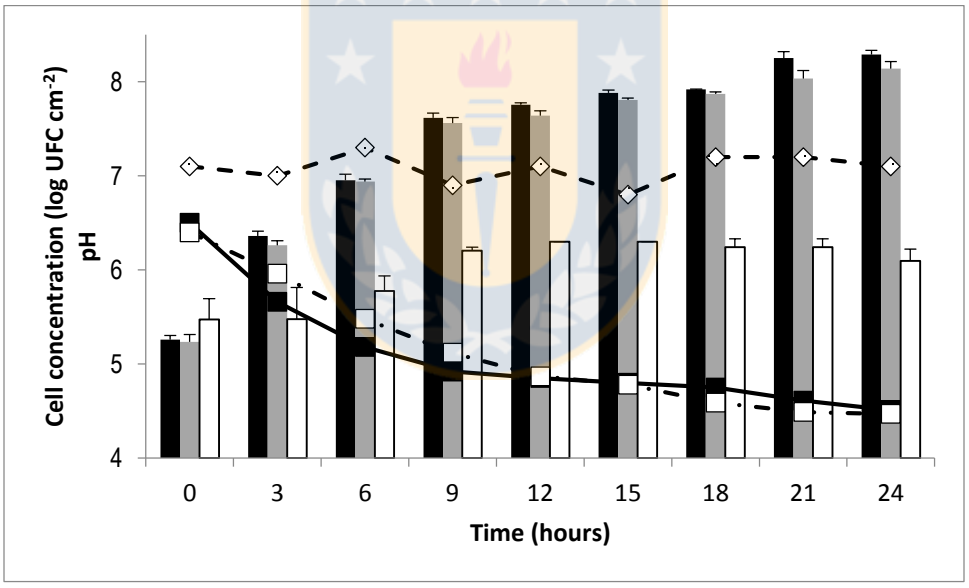

Figure 29: Evolution of the cell concentrations of *L. innocua* ATCC 33090 as measured on Aloa broth for the monoculture (black) and co-culture with *P. acidilactici* LPS28 (white)and LPS26 (grey) in smoked salmon at 15°C for 24 h; pH in the monoculture (◇) and in the co-culture with *P. acidilactici* LPS28 (□) and LPS26 (■).

Chapter 5: Discussion

Many *Pediococcus acidilactici* strains with the ability to produce antimicrobial peptides had been studied and they had attracted the interest for the use of either the cultures or their products as protective cultures or biopreservatives, respectively in food (Nieto-Lozano et al., 2009; Papagianni and Anastasiadou, 2009; Patil et al., 2009; Cintas et al., 1995; Piva and Headon, 1994).

The inhibition spectrum of bacteriocins of *Pediococcus* spp is very wide and different between the strains of the genus. It is very difficult to compare their potencies because each pediocin already studied was proven against different series of bacteria. *P. acidilactici* LPS28, *i.e.*, presented a pronounced antilisterial activity, acting against all the *Listeria* spp studied. Also, it acts against strains related to the producer and other lactic acid bacteria, no presenting activity against *Staphylococcus* spp. All the bacteriocin of *Pediococcus* spp reported presented antilisterial activity, some of them inhibits *Staphylococcys aureus* strains and most, one or more LAB (Neera et al., 2013; Abbasiliasi et al., 2012; Sivakumar et al., 2010; Mandal et al., 2008; Piva and Headon, 1994; Chikindas et al, 1993).

To characterize the antimicrobial peptide, the effects of the pH, proteolytic enzymes and of treatments at different temperatures were displayed. With respect to proteases effect, the antimicrobial peptide was inactivated by proteinase K but not by trypsin, in agreement with bacteriocins such as pediocins SA-1, NV-5 and PD-1 (Mandal et al, 2008; Anastasiadou et al., 2008; Green et al., 1997). Also, this three bacteriocions form *P. acidilactici* strains as the peptide from this study, showed to be heat stable remaining their antibacterial activity after different treatments at 100°C or 121°C. The prior is a characteristic of most of class IIa bacteriocins (Papagianni and Anastasiadou, 2009). The results of pH effect on antimicrobial activity showed similar tendency as all class IIa maintaining their activity at pH from 2 to 10, although in some cases there is a loss of activity in the high alkaline conditions due to alkaline lysis (Abbasiliasi et al., 2012; Mandal et al., 2008;). These data can confirm that the protein in study correspond to a bacteriocin, because of their protein nature by losing activity when it was treat with proteolytic enzymes, and a class IIa pediocion because of being heat stable, and present an antilistial activity.

Many pediocins (PD-1, SA-1, AcH/PA-1, SJ-1, AcM, F, PA1.0, NV-5, and others) had been purified and obtained their molecular weight produced by different pediococci strains (Anastasiadou et al., 2008; Mandal et al., 1998; Osmanağaoğlu et al., 1998; Elegado et al., 1997; Green et al., 1997; Schved et al., 1993; Nieto-Lozano et al., 1992; Gonzalez and Kunka, 1987). The purification methods and obtaining of the molecular weight used for the pediocins already identified are different, so the differences in the exact molecular weight can vary. Two of them, NV-5 and PA1.0, showed molecular weights higher than 10 kDa. The size of the others bacteriocins are lower than 5kDa. The best known pediocin AcH (or pediocin PA-1) produced by a *P. acidilactici* (Nieto-Lozano et al., 1992) isolated from commercial starter cultures present a MW of 4646 Da. The meat isolated *P.acidilactici* NRLLB5627 produced pediocin SA-1 (Anastasiadou et al., 2008) that showed a band on Tricine-SDS-PAGE between 3-4 kDa, weight that was confirmed by ESI-MS defining the exact MW of 3.66 kDa. Others, like pediocin F produced by *P.acidilactici* F isolated from fermented sausage was obtained by dialysis and presented a MW of 4.46 kDa. The smallest pediocin identified at the moment is produced by P. acidilactici H (Bhunia et al., 1987), a fermented sausage isolate, that present a MW of 2.7 kDa obtained by a special SDS-PAGE technique. The antimicrobial peptide of this study was purified by solid phase extraction and HPLC, and the molecular weight was first obtained by Tricine-SDS-PAGE and confirmed by LC-MS. The final result showed 3 different bands on the gel when it was carry out with urea (near 3, 5 and 7 kDa), comparing with the LC-MS, that showed only one pick of 2247 Da, we can verified that the smallest band correspond to the peptide, and the other can be aggregates of it.

The characteristics, inhibition spectrum and molecular weight of the different bacteriocins of *P.acidilatici* strains studied until now vary in agreement to the nature of the strain and where was isolated, considering that most of the fermented sausage isolated presented a MW near 4 kDa. Only pediocin ET34 produced by a salmon isolate *P.acidilactili* is already studied, and its molecular weight was obtained by SDS-PAGE and it is 3.5 kDa (Tomé et al., 2009). This size is not exact, indeed is similar to the antimicrobial peptide of *P. acidilactici* LPS28.

Also the capacity that *P. acidilactici* LPS28 showed to permeabilize the membrane of *L.innocua* ATCC 33090 is a propriety that class IIa bacteriocins have, because they

permeabilize the cytoplasmic membrane through pore formation by insertion of the C-terminal regions into the membrane (Drider et al., 2006).

Environmental conditions play an important role in lactic acid bacteria growth and bacteriocin production (Zhang et al., 2012; Papagianni and Anastasiadou, 2009; Mandal et al., 2008; Mataragas et al., 2003; Messens et al., 2003; Leroy and De Vuyst, 2002; Aasen et al., 2000; De Vuyst et al., 1996). The results obtained from this study showed that temperature, pH and culture medium affects both growth and bacteriocin production by *Pediococcus acidilactici* LPS28. The growth of *P. acidilactici* LPS28 resulted in primary metabolite kinetics of bacteriocin production, paralleling cell growth, as is usually observed for LAB (Leroy and De Vuyst, 1999) and remained constant up to the end of the fermentation. Several bacteriocin are produced during the exponential growth phase with a maximum in the middle or at the end of this phase or at the beginning of the stationary phase (De Vuyst et al., 1996; De Vuyst and Vandamme, 1992; Parente et al., 1994).

Besides a somewhat moderate effect on cell growth, the composition of the medium applied clearly affected bacteriocin production. The fact that the bacteriocin titre was maximal in LWP, a medium relatively richer in nitrogen sources and sugars than the other media, suggests that the bacteriocin production is a nutrient-dependent process. The latter is supported by Kemperman et al. (2003) and Kawai et al. (2003) that indicates that bacteriocin production by pediococci is increased in complex medium rich in nitrogen. Despite MRS broth is the most common commercial medium for lactic acid bacteria, the results both in growth and production of the antimicrobial weren´t the best for *P. acidilactici* LPS28, as Ivanova et al. (2002) reported, and the suggested modified MRS without phosphates and Tween, gave higher cell concentration and bacteriocin titers insinuating an effect of Tween on pediocin production as the case of pediocin A (Papagianni and Anastasiadou, 2009). Effects of sugar type on specific bacteriocin production, *i.e.*, regardless of cell growth, have been described previously for enterocin 1146 (Parente and Ricciardi, 1994) and for amylovorin DCE 471 (Leroy et al., 2006). Besides sugars, other medium components may also influence specific bacteriocin production, including the overall complex nutrients (Verluyten et al., 2004a ; Leroy and De Vuyst, 2001; Aasen et al., 2000) spices (Verluyten et al., 2004b), salts (Leroy et al. 2003; Matsusaki et al. 1996), and phosphates (Daba et al. 1993).

The optimum pH for bacteriocin production by *P. acidilactici* LPS28 was 6.0, coinciding with optimum pH for growth. The antimicrobial was produced at all the pH studied (4.5 - 8.5) and the bacteria growth reaching cell concentration since 7 Log CFU ml^{-1} in the range studied. It had been reported that *Pediococcus acidilactici* ssp could growth at pH between 3.5 and above 8.0 (Zhang et al., 2012; Papagianni and Anastasiadou, 2009). *Pediococcus acidilactici* PA003, for example, presented an optimum pH for growth of 6.0, which differed to the one for pediocin production that was 6.5. Other LAB showed similar behavior, presenting different ideal pH for growth and production but always in range of 5.5 – 6.5, as *Lactobacillus sakei* CCUG42687 (Aasen et al., 2000), *Leuconostoc mesenteroides* L124 and *Lactobacillus curvatus* L442 (Mataragas et al., 2003), and *Lactobacillus curvatus* LTH 1174 (Messens et al., 2003). In comparison, the production of sakacin K presented a less broad range of pH for its production, only between 4.5 and 5.5 (Leroy and De Vuyst, 1999). Also, initial and terminal pHs of fermentation greatly influenced pediocin production, that's why fermentations with control and uncontrolled pH were studied, obtaining similar growth and production curves but with lower final concentrations in the second case. Mataragas et al. (2003), also studied this effect, and they obtained that at uncontrolled pH because of the low pH obtained, the cell growth stopped and accordingly the bacteriocin production, so final high biomass and bacteriocin levels could not be obtained. Also, it has been studied that the pH decline rate and the final pH reached in the cultures appear to be critical factors in pediocin production. It has been shown for pediocins produced by *P. acidilactici* that production displays primary metabolite kinetics depending on the pH decline rate (Guerra and Pastrana, 2003; Ray, 1995).

The temperature influenced *Pediococcus acidilactici* LPS28 growth and bacteriocin production, but less pronounced in the latter. At 37 °C the higher cell concentration was obtained, but at 32 °C similar antimicrobial titers were obtained. Zhang et al. (2012) also reported different optimum temperatures for growth and pediocin production of 35 °C and 30 °C, respectively. The pediococci strain studied on this work could growth in the range of temperatures studied (25 – 37 °C) and be active metabolically. Zhang et al., (2012) reported that *Pediococcus acidilactici* spp can growth that temperatures between 25°C and 40°C, and optimum temperature for production was 30°C for other pediocins studied (Anastasiadou et al., 2008; Ray, 1995). Krier et al. (1998) indicates that the lower the temperature, the higher

the volumetric production, in special for mesenterocin 52A produced by *Leoconostoc mesenteroides*, and indicates that bacteriocin production is stimulated by temperatures unfavourable for growth, particularly the low temperatures. Thus, under uncontrolled pH conditions, a lower temperature coincided with a higher maximum bacteriocin production, a result also obtained by De Vuyst et al. (1996) with the bacteriocin from *Lactobacillus amylovorus*. On the other hand, Meera and Charitha Devi (2012) showed a significant reduction in the bacteriocin production as the temperature was increased indicating that growth temperature seems to play an important role in bacteriocin activity.

Different results had been reported and indicated that optimum fermentation conditions as temperature or pH or fermentation medium for the production of bacteriocins differed within the producing strains or the bacteriocin produced.

Encapsulation of bacteriocingenic lactic acid bacteria present many advantages for its use in the food industry by providing probiotic living cells with a physical barrier to resist adverse environmental conditions, prevention of interfacial inactivation, stimulation of production and excretion of secondary metabolites, and continuous utilization (Nazzaro et al., 2012; Burgain et al., 2011). Encapsulating *Pediococcus acidilactici* LPS28, bacteriocin production increased with respect to production in batch fermentations as free cells, and its concentration depends on the percentage of alginate used and the fermentation broth.

Low bacteriocin produced by lactic acid bacteria levels had been reported during batch fermentation, resulting in high production costs (Naghmouchi et al., 2008; Ivanova et al., 2002; Wan et al., 1995). Most authors had studied the bacteriocin production by *Pediococcus* ssp as free cells in batch or continuos fermentations (Naghmouchi et al., 2008; Nel at al., 2001; Cho et al., 1996; Huang et al., 1996; Daba el at., 1993; Liao et al., 1993). Naghmouchi et al. (1998) studied the effect of immobilization in repeated-cycle batch fermentations obtaining an increase of 1.5 log CFU g^{-1} after the first 16 h in MRS broth and a maximum bacteriocin titer of 4096 AU ml^{-1}. Meanwhile, Huang et al. (1996) and Cho et al. (1996), studied immobilized *Pediococcus* spp in continuos fermentations in MRS broth with maximum bacteriocin production of 1024 and 6400 AU ml^{-1}, respectively. Also, there are other authors that immobilized lactic acid bacteria and obtained similar results as in this study. Ivanova et al. (2002) encapsulated *E. faecium* 2000 and Sarika et al. (2012) immobilized *Lactobacillus plantarum* MTCC B1746 and *Lactococcus lactis* MTCC B440

showing that immobilization of lactic acid bacteria in calcium alginate beads made possible the increasing of bacteriocins production as a result of the increasing of biomass. In both studies, the viability of immobilized cells remained almost stable until the end of the fermentations in modified MRS broth. In this study, cells concentration of *Pediococcus acidilactici* LPS28 also remained constant during the 36 h of batch fermentation at 32°C.

Bacteriocin production by immobilized *Pediococcus acidilactici* LPS28 in batch fermentations in MRS broth starts after 6 h and reached a maximum of 4.33 x 10^4 AU ml^{-1} after 18 h and maintained constant up to the end of the fermentation. This bacteriocin titer is more than 7 times higher than the production of pediocins obtained by other authors (Naghmouchi et al., 2008; Cho et al., 1996; Huang et al., 1996), indicating an effectiveness much higher than other bacteriocins of *Pediococcus* spp, although it is difficult to compare because of different indicator strains and methods used. With to other bacteriocins, the production profiles with immobilized lactic acid bacteria indicates a higher delay in the protein production of more than 24 h bacteriocin titers lower than 10^4 AU ml^{-1} but cells maintained maximal production at the end of the fermentations process (Sarika et al., 2012; Ivanova et al., 2002; Scannell et al., 2000; Bhugaloo - Vial et al. 1997). The latter could be explained by alginate beads had a protective role separating the bacteriocin delivered to the medium from the proteolytic enzymes in the beads (Ivanova et al., 2002). Also, the immobilization conditions of higher cells density and substrate concentration could affect the bacterial metabolism related to the bacteriocins synthesis (Zezza et al. 1993). An finally, the most recent explanation is related to quorum sensing mechanisms, because it had been reported that most of class II bacteriocins has clearly shown to be regulated by this mechanism (Rizzelo et al., 2014), and in immobilization cases, dense growth of cells and limited diffusion of signalling molecules would increase bacterial communication. This could be the reason there some antimicrobial compounds that can be synthase on solid but not in liquid media (Braem et al. 2014; Maldonado-Barragán et al., 2009; West and Warner 1988).

As the calcium alginate beads are porous matrix with a molecular weight cut-off of approximately 20kDa (Tanaka et al., 1984) the bacteriocins released to the surround media and also some bacteria leaks out from the bead and grow in the medium as free cells

(Westman et al., 2012). The latter was checked on this study obtaining final concentration of cells in the liquid media of 10^6 CFU ml^{-1}.

Twenty four repeated cycle batch fermentation with immobilized *P. acidilactici* LPS28 were performed for four days. Cells concentration in the beads remained constant after the assay but free cells in the medium and bacteriocin titers decreased between cycles. Different results were obtained by Sarika et al. (2012) were cycles were performed for 7 days, and bacteriocin production increased cycle by cycle and by Ivanova et al. (2002) were after 96 h cycles, bacteriocin concentration was constant. This can be explained by a reduction on free cells release and increase of cells density on the beads.

The medium used for batch fermentation of 36 h with immobilized *P. acidilactici* LPS28 presented an important effect on the final bacteriocin titer obtained. Lactase-treated whey permeate (LWP), was the medium where the higher bacteriocin concentration was reached, followed by modified MRS. The worst results were obtained with WP what was expected because lactose is not the principal substrate for *Pediococcus* ssp metabolism (Naghmouchi et al., 2008). Also, others authors (Ivanova et al., 2002 and Sarika et al., 2012) used mMRS for fermentation with immobilized cells because they suggested that phosphates act disintegrating calcium alginate beads. In this study higher bacteriocin production was obtained with mMRS, but also high bacteriocin titer was reached with MRS. Naghmouchi et al. (2008) reported no difference on the final protein production on MRS and SWP.

The effect of the alginate percentage on bacteriocin production was studied in batch fermentation in LWP with immobilized *P. acidilactici* LPS28 obtaining higher titers at 2 and 2.25%. No references of this effect was found on production of lactic acid and on the survival of encapsulated lactic acid bacteria. Idris and Suzana (2006) reported that at low sodium alginate concentration resulted in very soft beads easily to be broken because of their low mechanical strength, resulting in bacteria leakage from the beads, and whilst increased the sodium alginate to above 2% hardened the beads, causing diffusion problems and the bacteria do not get enough nutrients as the substrate has difficulty in diffusion through the beads. Mandal et al. (2006) observed that the viability of encapsulated *L. casei* NCDC-298 cells improved with increasing alginate concentration in simulated gastric pH and intestinal bile salt solutions, but no metabolite production was studied. The latter can be expected because lower bacteria leakage may occur.

To describe the average diffusion that occurs in any position within the calcium alginate beads, the coefficient of effective diffusion was obtained. The value calculated for pediocin in the matrix was of 2.5 x 10^{-5} cm^2 h^{-1}. No information about bacteriocins already described had been reported in previous studies. In the review article of Pilkington *et al.* (1998), values for substrates as glucose, lactose, sucrose in calcium alginate beads are mentioned in the order of 2.0 -4.0 x 10^{-4} cm^2 h^{-1}. Also, Weber *et al.* (2009), calculated diffusion coefficients in hydrogels for different proteins, and the values depended on the molecular weight of the protein. The lowest molecular weight protein studied was the insulin (5700 g mol^{-1}) with a D_e of 1.3 x 10^{-4} cm^2 h^{-1}. Considering the latter, the coefficient obtained is a bit higher than expected, but experimental error can be achieved.

Continuous fermentation of lactic acid bacteria is currently employed to produce a number of microbial metabolites, in special lactic acid (Norton et al., 1994; Schepers et al., 2006). Continuous production of bacteriocins had being studied for lacticin 3147 (Scanell et al., 2000), brevicin (Wan et al., 1995), divercin (Bhugaloo-Vial et al., 1997), nisin (Wan et al., 1995; Scanell et al., 2000; Liu et al., 2005) and pediocin (Wan et al., 1995). Few investigations of continuous fermentations with immobilized *Pediococcus acidilactici* cells reported to date. Wan et al. (1995) studied *Pediococcus acidilactici* PO2 and the production of pediocin and obtained equivalent concentrations to those obtained in free-cell fermentations. The latter was similar to the results obtained in the present study. With respect to the effect of the dilution rate, Norton et al. (1994) postulate that an immobilized cell system for production of primary metabolites operates ideally at high dilution rates, because of the great availability of the substrate and relatively low concentration of inhibiting products. The highest lactic acid production was obtained at dilution rates higher than 1 h^{-1}. Similar results were obtained from Bhugaloo-Vial et al. (1997). Others as Liu et al. (2005) reported the higher productivity at 0.2 h^{-1}. In the present study, the highest dilution rate tested was 0,6 h^{-1}, and the highest pediocin volumetric productivity was reached at 0,25-0,3 h^{-1}.

The biomass from the calcium alginate beads, in the continuous fermentation reached it maximum at higher dilatation rates, and biomass leakage to the supernatant increased with it. The biomass concentration was always about 100 times higher in the beads than in the supernatant. The latter is comparable to Norton et al. (1994), where 50 times higher biomass concentration was obtained in the bead than in the supernatant. Then, cell leakage from the

beads is slow up to D = 0,3 h^{-1}. This could be important because it indicates that the removal of cells from broth as required in free cells fermentations process would not be needed prior to purification or concentration of bacteriocins.

Also, growth and bacteriocin production by *Pediococcus acidilactici* LPS28 differed between cells that were cultivated in liquid medium and cells that were immobilized in alginate-based gel systems. Planktonic growth resulted in primary metabolite kinetics of bacteriocin production, paralleling cell growth, as is usually observed for LAB (Leroy and De Vuyst, 1999). Besides a somewhat moderate effect on cell growth, the composition of the medium applied clearly affected bacteriocin production. In the immobilized alginate systems, cells generally grew slower and to lower cell counts than the free cells but specific bacteriocin production was nevertheless enhanced, agreeing with previous studies that found an inverse relationship between optimal growth and bacteriocin production (Krier et al., 1998; Leroy and De Vuyst, 1999a; Leroy et al., 2003; Delgado et al., 2007). The specific local conditions of cell density, substrates, and products in an immobilized system could affect bacterial metabolism related to the synthesis and release of bacteriocins (Zezza et al., 1993). Enhanced production due to cell immobilization has also been reported by for the production of bacteriocin by *Enterococcus faecium* A2000 in modified MRS (Ivanova et al., 2000) and brevicin by *Lactobacillus brevis* VB286 (Bhugaloo et al., 1997). In contrast, nisin production by alginate-immobilized *Lactococcus lactis* subsp. *lactis* NZ1 was lower than with free-cell cultures (Zezza et al., 1993). In some cases, bacteriocin production does only occur with bacteria growing on a solid system and not in liquid media, as for plantaricin B and nukacin L217 produced by *Lactobacillus plantarum* NCDO 1193 (West and Warner, 1988) and *Staphylococcus chromogenes* L217 (Braem et al., 2014), respectively. A possible explanation would be related to the fact that bacteriocins are regulated by quorum-sensing mechanisms, whereby dense growth of immobilized cells and limited diffusion of signalling molecules would increase bacterial communication (Rizzello et al., 2014). Also, the incorporation of bacteria into solid surfaces may cause changes in the expression of relevant genes involved in bacteriocin regulation.

For most experiments with immobilized cells, bacteriocin activity only started after the stationary phase was reached. This effect was less clear in LWP, for reasons yet unknown. Although this shift in activity could be due to altered bacteriocin production kinetics, a more

straightforward explanation may be related to diffusion limitations that govern the release of the bacteriocin into the supernatant. Although strong strain-dependent variations in the onset and shutdown of bacteriocin production by LAB have been found (Leroy and De Vuyst, 2002; Delgado et al., 2007), only few studies have reported on bacteriocin production during the stationary phase. Jiménez-Diaz et al. (1993) described the production of two bacteriocins by *L. plantarum* LPCO10 in liquid MRS during different stages of population development. Whereas a first bacteriocin was produced during the active growth phase, a second one with different activity spectrum only appeared during the late stationary phase. Biswas et al. (1991) reported that about 60 % of pediocin activity by *P. acidilactici* H in MRS medium was produced during the first 8 h of growth, while the final 40 % was generated during the stationary phase.

Finally, the use of *P. acidilactici* LPS28 as a potential bioprotective culture for smoked salmon was explored. Promising results were obtained as the strain could grow well in the product, displaying growth behaviour that matched earlier observations with lactic acid bacteria (Tahiri et al., 2009; Duffes et al., 1999; Joffraud et al., 2006), although lower or higher Δ log increases may be possible in animal muscle products (Katla et al., 2001; Tomé et al., 2008; Nieto-Lozano et al., 2010). Also, the growth of *P. acidilactici* LPS28 was not affected by the presence of *L. innocua* ATCC 33090. The latter strain was able to grow well in smoked salmon, reaching concentrations greater than 10^7 CFU cm^{-2} after merely one day of storage (both at 4°C and 15°C). Other authors reported similar rapid listerial growth in fish (Katla et al., 2001; Nilsson et al., 2004; Duffes et al., 1999; Tomé et al., 2008) or other food matrices (Nieto-Lozano et al., 2010; Foulquié Moreno et al., 2003). In the presence of *P. acidilactici* LPS28, however, a bacteriostatic effect on *L. innocua* ATCC 33090 was found, resulting in a log difference of about 3 log CFU cm^{-2} compared to the control experiments (with *L. innocua* ATCC 33090 as a monoculture or in a co-culture with the non-bacteriocinogenic *P. acidilactici* LPS26). This effect was in the order of the results obtained during experiments with *C. divergens* M35 in smoked fish (Tahiri et al., 2009) or with other lactic acid bacteria in liquid media (Todorov et al., 2011), but higher than with co-cultures using *C. divergens* V41 and *P. acidilactici* ET34, where only 0.5 to 1.0 log decreases were obtained (Vaz-Velho et al, 2005; Tomé et al, 2008).

The reduction on *L. innocua* cell counts most likely was linked to the release of bacteriocin by *P. acidilactici* LPS28, as supported by the demonstration of *in situ* production and by the comparison with the non-bacteriocingenic but equally acidifying *P. acidilactici* LPS26. In general, *in situ* bacteriocin production in fish and fish products depends on the strain and its competitiveness in the food matrix. Moreover, interactions with the matrix as well as with the target bacteria and the background microbiota need to be taken into account (De Vuyst and Leroy, 2007). Although several authors have shown that bacteriocins of lactic acid bacteria can act against *Listeria* spp. in smoked salmon (Duffes et al., 1999; Katla et al., 2001; Nilsson et al., 2004; Brillet et al., 2005; Ghalfi et al., 2006; Vaz-Velho et al., 2005; Ye et al., 2008; Datta et al., 2008), only one study focussed on a bacteriocin produced by a *Pediococcus* sp. (Tomé et al., 2008).

Chapter 6: Conclusions

- The bacteriocin produced by *Pediococcus acidilactici* LPS28 presents properties of a pediocin-like class Ila bacteriocin, because of the strong antilisterial activity and heat stability. It thus offers potential for biopreservation.
- *Pediococcus acidilactici* LPS28 immobilization in 2% calcium alginate spheres and their latter batch fermentation in LWP medium present the higher bacteriocin production reaching a final concentration of 5.33 x 10^4 AU ml^{-1}. Also, the cells concentration in the spheres remained constant during repeated cycle batch fermentations demonstrating a high stability of the beads. Considering the high production and the stabilty of the beads its may facilitate the development of large-scale industrial process for other lactic acid bacteria bacteriocins.
- Immobilization enhances BLIS production, leading to up to six times higher specific BLIS production values and a shift of BLIS activity towards the stationary phase. Effects may be due to altered production kinetics or to diffusion effects.
- The evolution of bacteriocin concentrations over time is dependent on whether the producing cells are in planktonic or immobilized state. Further studies are required to investigate if the observed change in kinetics is to be situated on the level of the molecular regulation involved in bacteriocin production. Also, diffusion effects will have to be analyzed in sufficient detail, as they may affect the mobility of both the bacteriocin and potential induction factors. Finally, implications of the textural character of food matrices on bacterial growth and subsequent production of bacteriocin will have to be taken into account. This may be particularly the case when LAB are added as bioprotective cultures to gel-like food systems, as would be the case for smoked salmon.
- Alginate systems can serve as a more realistic model system for gel-like food systems (incl. meat, fish, and cheese) than liquid media.

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