UNIVERSITY OF SÃO PAULO

FACULTY OF PHARMACEUTICAL SCIENCES

Department of Biochemical and Pharmaceutical Technology

Biotechnological production and application of antimicrobial biomolecules by *Lactobacillus plantarum* in milk whey

Sabrina da Silva Sabo

Thesis to obtain the degree of Doctor

Adviser: Prof. Dr. Ricardo Pinheiro de Souza Oliveira

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SABO, S.S. **Produção biotecnológica e aplicação de biomoléculas antimicrobianas por *Lactobacillus plantarum* cultivado em soro de leite.** 2017. 197 p. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2017.

Bacteriocinas são peptídeos antimicrobianos sintetizados por diversas bactérias. Em particular, as bacteriocinas produzidas por bactérias ácido-láticas têm recebido crescente destaque devido sua ampla aplicabilidade e também por serem produzidas por micro-organismos seguros para o consumo humano. Inúmeros estudos relatam a produção de bacteriocina por diversas bactérias ácido-láticas, bem como a otimização da produção desta biomolécula utilizando fontes alternativas de substrato. Alguns destes estudos apontam o soro de leite/queijo como uma fonte potencial e de baixo custo para o cultivo de bactérias láticas e produção de biocompostos, tais como bacteriocinas. Diversas pesquisas se referem à produção de bacteriocinas, principalmente a nisina. Por outro lado, a produção de bacteriocinas por cepas de *Lactobacillus plantarum* ainda é limitada. Neste âmbito, o presente trabalho estudou, primeiramente, o crescimento e produção de bacteriocina pela cepa *L. plantarum* ST16Pa quando cultivada em caldo *Man, Rogosa e Sharpe* (MRS) com e sem suplementação de inulina sob condições aeradas e sem agitação. Os resultados indicaram que este micro-organismo tem maior crescimento quando cultivado em aerobiose, entretanto, os maiores valores de atividade da bacteriocina produzida foram obtidos em cultivos não agitados. Ainda utilizando MRS para o cultivo desta cepa, investigou-se a estabilidade e partição da bacteriocina em sistema aquoso de duas fases do tipo PEG/NaPA/electrólitos. Após 1 h submetida à temperaturas entre 50 °C e 80 °C e pH entre 3,0 e 8,0, a bacteriocina produzida permaneceu estável. Além disso, o sistema de partição proposto demonstrou ser uma alternativa econômica para recuperação e purificação desta biomolécula. Motivados pela tendência do uso de subprodutos industriais para a produção de biocompostos de alto valor agregado, investigou-se o desempenho do soro de leite/queijo como substrato alternativo para o crescimento de *L. plantarum* ST16Pa e a consequente produção de biomoléculas antimicrobianas por esta cepa. O estudo foi divido em duas etapas, sendo a primeira desenvolvida utilizando soro de leite em pó suplementado de acordo com o delineamento experimental Plackett & Burman, com 12 ensaios e mais 4 pontos centrais. Embora o delineamento proposto não tenha sido a ferramenta estatística ideal para alcançar a otimização da produção de bacteriocina ST16Pa, através do mesmo foi possível obter uma formulação, a qual gerou maiores halos de inibição quando comparados com resultados prévios reportados por outros autores utilizando a mesma cepa cultivada em soro de queijo. Posteriormente, ainda utilizando *L. plantarum* ST16Pa, investigou-se a produção de bacteriocina e lactato em soro de queijo fresco previamente hidrolisado por Flavourzyme ou Lactozym. Os cultivos foram otimizado através da suplementação deste meio de cultura com diferentes concentrações de farinha de soja, os quais foram incubados sob condições aeróbicas e microaerofílicas/anaeróbicas. Os maiores valores de atividade da bacteriocina (7367,23 AU/mL) e rendimento de lactato ($Y_{lactate/lactose}$ = 1,39 g/g) foram obtidos sob condições anaeróbicas utilizando 10 g/L de farinha de soja para suplementar o soro de queijo hidrolizado por Flavourzyme durante 3 h. Por fim, o sobrenadante resultante deste cultivo foi aplicado sobre a superfície de filés de peito de frango artificialmente contaminados por *Enterococcus faecium* 711, demonstrando eficiência como bioconservante durante 7 dias de armazenamento sob refrigeração.

Palavras-chave: soro de leite/queijo, *Lactobacillus plantarum*, bacteriocina, suplementação, otimização, bioconservante

ABSTRACT

SABO, S.S. **Biotechnological production and application of antimicrobial biomolecules by *Lactobacillus plantarum* in milk whey.** 2017. 197 p. Thesis (Ph. D.) – Faculty of Pharmaceutical Sciences, University of São Paulo, 2017.

Bacteriocins are antimicrobial peptides ribosomally synthesized by various bacteria. In particular, the bacteriocins produced by lactic acid bacteria have recently received great attention due to their wide applicability and also because they are produced by microorganisms safe for human consumption. There are numerous studies reporting bacteriocin production by different lactic acid bacteria, as well as its optimization by using alternative substrate. Some of these studies point to milk/cheese whey as a potential low-cost source for the cultivation of lactic acid bacteria and the production of biocomposites, such as bacteriocins. Several researches refer to the production of bacteriocins, mainly nisin. On the other hand, the production of bacteriocins by *Lactobacillus plantarum* strains still limited. In this context, the present work first studied the growth and production of bacteriocin by *L. plantarum* strain ST16Pa when cultivated in Man, Rogosa and Sharpe (MRS) broth with and without inulin supplementation incubated under shaken and stationary conditions. The results indicated that this strain has higher growth when cultured in aerobiosis; however, the highest bacteriocin activity is obtained in stationary condition. The stability and partitioning of bacteriocin from the fermented MRS broth of *L. plantarum* ST16Pa in the PEG/NaPA/electrolytes aqueous two-phase systems was also studied. After 1 h in temperatures from 50 °C to 80 °C and pH from 3.0 to 8.0, the produced bacteriocin remained stable. In addition, the proposed partition system has proved to be an economical alternative for the recovery and purification of this biomolecule. Motivated by the trend in use industrial byproducts aiming the production of high value-added biocomposites, we investigated the performance of milk/cheese whey as alternative substrate for *L. plantarum* ST16Pa growth and for the production of antimicrobial biomolecules by this strain. For this purpose, the study was divided in two steps, being the first developed using whey powder supplemented according to the Plackett & Burman experimental design, with 12 trials and 4 central points. Although the proposed design was not the ideal statistical tool to optimize the bacteriocin ST16Pa production, through it was possible to obtain a formulation, which resulted in greater inhibition zones when compared with previous date reported by other authors using the same strain cultured in cheese whey. Later, still using *L. plantarum* strain ST16Pa, we investigated the production of bacteriocin and lactate in fresh cheese whey previously hydrolyzed by Flavourzyme or Lactozym. The cultures were optimized by supplementing this medium with different concentrations of soybean flour, which were incubated under aerobic and microaerophilic/anaerobic conditions. The highest values of bacteriocin activity (7,367.23 AU/mL) and lactate yield ($Y_{lactate/lactose}$ = 1.39 g/g) were obtained under anaerobic conditions using 10 g/L of soybean flour to supplement cheese whey prehydrolyzed with Flavourzyme during 3 h. Finally, the cell-free supernatant resulted from this cultivation was applied on the surfaces of chicken breast fillets artificially contaminated with *Enterococcus faecium* 711, showing biopreservative efficiency during 7 days of storage under refrigeration.

Keywords: milk/cheese whey, *Lactobacillus plantarum*, bacteriocin, supplementation, optimization, biopreservative

CONTENTS

GENERAL INTRODUCTION

Due to the growing production of processed foods, the consumer is increasingly worried about the presence of chemical additives in their diet. For this reason, there is a trend to seek for foods that are considered "natural" or fresh, without the addition of chemical preservatives. This fact has stimulated researchers to search for effective natural preservatives. In addition, the resistance of pathogenic microorganisms to antibiotics has been considered one of the major public health problems as a result of the abusive use of these compounds in the treatment of human and animal diseases. Alternatively, lactic acid bacteria (LAB) have been widely used in food industry as a natural preservative because their ability to produce antimicrobial compounds, such as bacteriocins, organic acids, among others. In this context, this thesis deals with the main aspects of these biomolecules, being divided into five chapters written in the form of articles. The chapter I comprise a comprehensive literature review on LAB and production of bacteriocins, especially those produced by *Lactobacillus plantarum*, since this microorganism is the lactic acid bacterium chosen for the development of this work. The chapter II investigated the best cultivation conditions of *L. plantarum* ST16Pa in the recommended commercial media Man, Rogosa and Sharpe (MRS). An additional source of carbon (inulin) was added to these cultures in order to evaluate whether this prebiotic fiber promotes optimization of growth and bacteriocin production by the studied strain. Still using MRS as culture medium for growth and bacteriocin production by *L. plantarum* ST16Pa, in chapter III it was studied the stability and partitioning of bacteriocin in a novel inexpensive and stable aqueous two-phase polymer system (ATPPS) composed by poly(ethylene glycol) (PEG) and sodium polyacrylate (NaPA), mixing both polymers with sodium sulfate (Na_2SO_4) and ionic liquid choline cloride ([Ch]Cl). Coupled with the environmental aspect, in the chapters IV and V were investigated the applicability of whey as a fermentable substrate for cultivating *L. plantarum* ST16Pa and produce bacteriocin. Specifically, in the chapter IV a Plackett & Burmann experimental design was proposed to enhance the production of bacteriocin ST16Pa inhibition zone using whey powder supplemented with additional carbon and nitrogen sources, besides vitamin and variations in shaking process conditions. Regarding the chapter V, fresh cheese whey, previously hydrolyzed and supplemented with soybean flour, was used to produce bacteriocin and lactate and this coumpund were investigated about their antimicrobial efficiency as biopreservatives on fresh chicken meat.

GENERAL AND SPECIFIC OBJECTIVES

The main objective of this thesis was the biotechnological production of antibacterial biomolecules, especially bacteriocin, by *L. plantarum* ST16Pa using whey as an alternative substrate. For this, the following specific objectives were established:

I. Evaluate the bacteriocin production by *L. plantarum* ST16Pa in recommended commercial culture medium (i.e. MRS).
II. Evaluate the efficiency of whey (powder and fresh) as an alternative culture medium to grow the *L. plantarum* ST16Pa and achieve bacteriocin production by this strain.
III. Obtain a whey powder formulation, using a Plackett & Burman experimental design as a statistical tool, through supplementation with additional sources of carbon, nitrogen and vitamin, varying the agitation (rpm), which could improve the bacteriocin ST16Pa production.
V. Study the production of bacteriocin and lactate by cultivating *L. plantarum* ST16Pa using previously hydrolyzed fresh cheese whey supplemented with soybean flour as a fermentative substrate and investigate their antimicrobial efficiency as biopreservatives on fresh chicken meat.
VI. Evaluate and compare the fermentative parameters of *L. plantarum* ST16Pa when cultured in orbital shaker and bioreactor, using both culture mediums: whey powder formulation and hydrolyzed cheese whey.
VII. Propose suitable bacteriocin protocol purification, aiming a future industrial application in both the food and pharmaceutical areas.

CHAPTER I[1]

Overview of *Lactobacillus plantarum* as a promising bacteriocin producer among lactic acid bacteria

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ABSTRACT

Chemical preservatives have been traditionally used during the manufacturing of processed products. However, the continuous growing interest of consumers for fresh and natural products makes necessary to search for alternative compounds. In this context, food industries have been widely using lactic acid bacteria (LAB) as natural preservatives, due to their ability to produce antibacterial compounds such as bacteriocins. Similarly, pharmaceutical industries have improved the use of these bacterial peptides, with antibacterial activity, trying to reduce the indiscriminate use of antibiotics in food products for human and animal consumption. Among LAB, *Lactobacillus plantarum* can be adapted to various niches thanks to its ability to ferment a wide range of carbohydrates. Additionally, it can be used as starter culture in food fermentations and as an ingredient for probiotic foods, contributing to the organoleptic characteristics of foods at the same time prolonging the shelf-life and safety of these products. The amount of valuable substances obtained from *L. plantarum* species isolated from different ecological niches is also worth nothing, thus proving it to be one of the most important and versatile species among LAB.

Keywords: lactic acid bacteria, *Lactobacillus plantarum*, bacteriocins, plantaricin, purification of bacteriocins

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1. Introduction

Industrialized food has experienced in recent years an increase in the use of chemical additives in their formulations; however, consumers are becoming more and more worried about using these chemical additives in their diet. For this reason, there is a strong trend for seeking natural and fresh foods, free of chemical preservatives. This fact, associated with the growing demand for minimally processed foods, has encouraged the search for effective natural preservatives, among which, antibacterial compounds such as bacteriocins, fulfill these requirements (Castro, Palavecino, Herman, Garro, & Campo, 2011).

In order to control these facts, an alternative would be to use bacterial peptides with antibacterial activity, such as bacteriocins (Parada, Caron, Medeiros, & Soccol, 2007).

Bacteriocins are peptides or proteins synthesized within ribosomes and released into the extracellular medium by Gram-positive and Gram-negative microorganisms, although, those produced by lactic acid bacteria (LAB) have received greater attention, in recent years, due to their high potential for application in the food industry as natural conserving agents (Leroy & De Vuyst, 2004). Among LAB, the largest group is the genus *Lactobacillus*, which comprises more than 150 different species (Siezen et al., 2010). Some of those are associated with the promotion of health benefits to the host (Bosch et al., 2011). Among them, *L. plantarum* can be pointed out as an industrially important microorganism that can be found and isolated from dairy products and fermented foods such as sauerkraut, sourdough, sausages, cheeses, wines, olives and pickled vegetables from environments such as cow-dung, silage and from sewage; as well as from the human mouth, intestinal tract and stools (Hammes & Vogel, 1995; Parente, Ciocia, Ricciardi, Zotta, Felis & Torriani, 2010).

In addition, certain *L. plantarum* strains have probiotic properties, which have been used for the development of functional foods and potential oral vaccines (Parente et al., 2010). To carry out such probiotic activities, the strains should have the ability to produce substances such as bacteriocins, which offer advantages in colonization and competition in the gastrointestinal tract (Castro et al., 2011). Additionally, these compounds have bactericidal or bacteriostatic action on pathogenic bacteria, which includes important pathogens such as *Listeria monocytogenes*, *Clostridium botulinum* and *Staphylococcus aureus* (De Vuyst & Leroy, 2007). Recently, the food industry has shown an increasing interest in the use of bacteriocins as a replacement for chemical preservatives, as they are effective at low concentrations and, when added to food, do not alter its sensory quality (Zacharof, Coss, Mandale, & Lovitt, 2013).

In this context, the purpose of this work is to provide, as a first step, a general overview of LAB along with the bacteriocins produced by these strains, before addressing a detailed study of the *L. plantarum* species, itemizing the bacteriocins produced up to now, with the aspect concerning their biosynthesis, their main applications, mainly in the food and pharmaceutical industries, and the purification considered in order to obtain a deeper knowledge of these peptide bacteriocins.

2. General characteristics of LAB

The group of LAB associated with food includes 11 genera, *Carnobacterium*, *Enterococcus*, *Lactococcus*, *Lactobacillus* (by far the most investigate genus), *Lactosphaera, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Vagococcus,* and *Weissella* (Mogensen, Salminen, & O´Brien, 2003; Vries, Vaughan, Kleerebezem, & Vos, 2006).

These microorganisms are Gram (+), non-sporulating, catalase-negative, acid-resistant, $pH_{optimum}$ for growing between 4.0 and 4.5, anaerobic aerotolerant, $T_{optimum}$ for growing is 30 °C (mesophilic) or 42 °C (thermophilic) and can have different shapes, like rods (bacilli) and a sphere (coccus) (Todorov & Franco, 2010).

Lactobacillus plantarum is a facultative heterofermentative lactic acid bacterium, which ferments carbohydrates generally by the phosphoketolase pathway (PKP). The fermentation of pentoses (xylose, ribose) leads to the formation of pyruvate and acetyl-P and their subsequent conversion to lactate and acetate, respectively. Hexoses (glucose, fructose, mannose) in these bacteria can be converted to lactate, CO_2, and ethanol (Hammes & Vogel, 1995; Mayo et al., 2010; Todorov & Franco, 2010). In addition, its genome encodes all enzymes required for the glycolysis and phosphoketolase pathways (Kleerebezem, Boekhorst, Kranenburg, Van Molenaar, Kiupers, Leer, et al., 2003). *L. plantarum* has broad versatility, insofar as it can be found in many ecological niches, as well as in human and animal gastrointestinal tracts (Siezen et al., 2010). The ability to inhabit different niches is associated with its ability to ferment a variety of sugars (Prins, Botha, Botes, Kwaadsteniet, Endo & Dicks, 2010). They have developed very efficient transport systems, which enable them to quickly take up the necessary solutes (Salminen & Von Wright, 1993). Particularly, this flexible and adaptive behavior of *L. plantarum* is reflected by the relatively large number of regulatory and transport functions, including 25 complete sugar-phosphotransferase systems (PTSs) (Kleerebezem et al., 2003).

LAB are used in the food industry due to their ability to inhibit or reduce contamination by spoilage and/or pathogen microorganisms through the production of various antimicrobial compounds (Martinez, Balciunas, Salgado, et al., 2013). The acidification of food – mainly by lactic acid formation – is probably the primary factor in the inhibition of undesirable microorganisms. LAB reduce the pH to values close to 4 in these foods, which hinders the survival of microorganisms sensitive to acid medium. Such condition leads to the increase of the fermented product shelf life, when compared to the non-fermented one (Leroy & De Vuyst, 2004).

These bacteria are still used in the food industry for the development of organoleptic properties of fermented foods (Carminati, Giraffa, Quiberoni, Binetti, Suárez & Reinheimer, 2010; Todorov, LeBlanc, & Franco, 2012). This occurs due to the large number of glycolytic, lipolytic and proteolytic enzymes that transform some medium nutrients into compounds with sensory properties, which, in the end, gradually modify the structure and aroma of the fermented food (Todorov et al., 2012). In addition, LAB are also used as probiotic starter cultures (Carminati et al., 2010).

3. Bacteriocins from LAB

LAB-producing bacteriocins (antimicrobial peptides synthesized in ribosomes) kill bacteria at much lower concentrations than eukaryotic antimicrobial peptides, probably because they interact with a specific receptor present on target cells (Cotter, Hill, & Ross, 2005; Drider, Fimland, Héchard, McMullen, & Prévost, 2006). Some studies indicated that the 35d-plantaricin bacteriocin produced by *L. plantarum* 35d showed to be active against *Aeromonas hydrophila*. Meanwhile bacteriocins ST28MS and ST26MS, produced by *L. plantarum* isolated from syrup inhibited the growth of *Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* and other Gram-positive microorganisms (Messi, Bondi, Sabia, Battini, & Manicardi, 2001; Todorov & Dicks, 2005a).

The bacteriocin production often occurs during late log phase or early stationary phase, and is generally influenced by a quorum sensing mechanism or by any sign of stress (Martinez, Balciunas, Converti, Cotter, & Oliveira, 2013). They differ from the majority of antibiotics due to their molecular proteinaceous constitution, being rapidly degraded by proteases in the human digestive tract (Parada et al., 2007).

3.1. History of bacteriocins

The first report of an antibacterial substance was conducted in 1925, when André Gratia published an article regarding the inhibitory capacity of *E. coli* on other strains of the same species. The produced compound, considered as responsible for the inhibitory effect, was called "colicin", in reference to the producer microorganism (Collins, Cotter, Hill, & Ross, 2010). In 1928, the ability of certain *Lactococci* strains to exert inhibition on other LAB strains was reported, and later, in 1947, Mattick and Hirsh concentrated an inhibitory substance isolated from a strain of *Lactococcus lactis* subsp. *lactis*, termed nisin (Cotter et al., 2005). This bacteriocin was initially purified and marketed in 1953 in England and then, in 1969, was considered to be safe for use in food products by the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives. It was also in 1953 that the term "bacteriocin" was proposed for antimicrobial peptides produced by microorganisms (Reeves, 1965). In 1983, in Europe, nisin was added to the list of food additives and, in 1988, the American Food and Drug Administration (FDA) authorized its use in processed cheeses (Collins et al., 2010). Although nisin is the only bacteriocin approved by the FDA for use in foods, pediocin, attained from strains of *Pediococcus acidilactici*, *Pediococcus parvulus* and *L. plantarum* WHE92, has also been employed as a preservative in industrialized foods (Enan, Essawy, Uyttendaele, & Debevere, 1996; Wang & Wang, 2014). Indeed, there is a commercial pediocin – ALTA 2341®, produced by Quest International, Sarasota, Florida from *P. acidilactici* – that is used as a medium component for fermentation processes (Papagianni & Anastasiadou, 2009). As ALTA 2341® presented high inhibitory action on *L. monocytogenes*, the producer applied for its approval by FDA (Chen, Sebranek, Dickson, & Mendonca, 2004).

A remarkable achievement on bacteriocins was attained in 1994, when a strain of lantibiotic called plantaricin C from *L. plantarum* LL441, was isolated from ripening cheese (González, Arca, Mayo, & Suárez, 1994).

3.2. Classification of bacteriocins

In spite of bacteriocins from lactic acid bacteria (LAB) differing in their spectrum of activity and in biochemical and genetic determinants, some common characteristics allow them to be divided into four classes, based on primary structure, molecular weight, heat stability and molecular organization (Cotter et al., 2005; Heng, Wescombe, Burton, Jack, & Tagg, 2007):

• Class I (lantibiotics): consists of linear (type A) and globular (type B) peptides, with low molecular weight (< 5 kDa, with approximately 19 to 38 amino acids). They are post-translational modified peptides that contain unusual amino acids such as lanthionine and derivatives (Cintas, Casaus, Herranz, Nes, & Hernández, 2001; Drider et al., 2006; Todorov, 2009). Nisin is the first and the most well-known lantibiotic (Ghrairi, Chaftar, & Hani, 2012);

• Class II (non-lantibiotics): composed of thermostable peptides (< 10 kDa, with approximately 37 to 48 amino acids), which, according to Drider et al. (2006) are divided into three subclasses known as Class IIa (group of pediocin-like bacteriocins active against *Listeria* spp.), Class IIb (bacteriocins requiring the union of two peptides to completely exert antibacterial effect, e.g., lactocin G) and Class IIc (bacteriocins which have a covalent bond between the C and N terminal, resulting in a cyclic structure) (Balciunas et al., 2013);

• Class III: represented by thermolabile peptides of high molecular weight (> 30 kDa), such as helveticin J, acidophilucin A, and lactacin A and B (Heng et al., 2007);

• Class IV: composed of complex bacteriocins that contain carbohydrate or lipid moieties, in addition to the protein portion (Heng et al., 2007). However, Cleveland, Montville, Nes, & Chikindas (2001) proposed that these complexes are artifacts of partial purification and not a new class of bacteriocins.

Among all of these peptide bacteriocins produced by LAB, subclass IIa has arisen as one of the most interesting groups for use in food preservation (inhibiting the growth of Gram-positive food spoilage and pathogenic bacteria such as *Bacillus cereus*, *Clostridium perfringens*, *S. aureus*, and *L. monocytogenes*) as well as in medicine (as antibiotic complements in treating infectious diseases or as antiviral agents) (Drider et al., 2006). Some bacteriocins produced by *L. plantarum* have been ascribed to this group, including plantaricin 423 produced by *L. plantarum* strain 423. This bacteriocin was able to inhibit the growth of *L. monocytogenes* in ostrich salami meat (Dicks, Mellett, & Hoffman, 2004).

Other plantaricins, such as EF, JK, NC8 and J51, were cataloged into the two-peptide bacteriocins (subclass IIb) (Anderssen, Diep, Nes, Eijsink, & Meyer, 1998; Diep, Havarstein,

& Nes, 1996; Diep, Straume, Kjos, Torres, & Nes, 2009). Their activity depends, by definition, on the complementary action of two different peptides, where their cationic nature is essential, facilitating the initial contact between bacteriocins and the negatively charged membranes via electrostatic interactions (Diep et al., 2009).

Finally, plantaricin A, a one-peptide bacteriocin without post-translational modifications, is included in subclass IIc (Diep et al., 2009). The antimicrobial spectrum of plantaricin A is relatively narrow, comprising mainly diferente *Lactobacillus* species (*Lactobacillus casei*, *Lactobacillus sakei*, *Lactobacillus viridescens* and *L. plantarum*). The antimicrobial activity of plantiricin A was 10-100 fold lower than plantiricins EF and JK (Anderssen et al., 1998).

3.3. Biosynthesis and mode of action of bacteriocins

According to Drider et al. (2006), at least four genes are needed for the production and secretion of bacteriocins. In particular, they are (i) the structural bacteriocin gene, encoding a prebacteriocin; (ii) the immunity gene, encoding an immunity protein that protects the bacteriocin producer from its own bacteriocin; (iii) a gene encoding an ABC (ATP-binding cassette) transporter necessary for secretion; and (iv) a gene encoding an accessory protein of unknown function. Bacteriocins can result from the expression of a gene located at the chromosome (plantaricin ST31 for example) or at a plasmid (plantaricin 423 for example) (Reenen, van Dicks, & Chikindas, 1998; Todorov, 2009; Todorov et al., 1999). However, when two bacteriocins are produced by a strain one can be chromosomal, such as carnobacteriocin BM1, and the other from the plasmid, such as carnobacteriocin B2 (Todorov, 2009). Of course, there are situations in which two or more bacteriocins can only originate either from the chromosome or plasmid (Todorov, 2009).

Most class II bacteriocins are synthesized primarily in the form of a pre-peptide or a biologically inactive pre-bacteriocin. This compound contains a sequence from 18 to 27 amino acids presenting two glycines at the N-terminus. This sequence has the function of preventing the bacteriocin to be activated inside the producer cell and serves as recognition signal for the transport system involving the ABC transporter proteins and accessory protein (Nes, Diep, Havarstein, & Brurberg, 1996; Savadogo, Ouattara, Bassole, & Traores, 2006). The two glycines present in the sequence are responsible for recognition by the pre-bacteriocin transport system (Moll, Konings, & Driessen, 1999). After recognizing the pre-peptide, the leader amino acid sequence of bacteriocin is removed and then the active

peptide/bacteriocin is secreted into the extracellular medium (Ehrmann, Remiger, Eijsink, & Vogel, 2000).

Regarding the mode of action, different mechanisms have been proposed for bacteriocins. Such mechanisms directly depend on factors related to bacterial species and their growth conditions, bacteriocin dose employed and purification degree (Parada et al., 2007). In particular, these mechanisms can promote a bactericidal effect, with or without cell lysis, or bacteriostatic, inhibiting cell growth (Cintas et al., 2001). Usually, pore formation – which results in the variation of the cytoplasm membrane potential due to the hydronium ion exchanging between the inner and outer membrane surfaces – is the main mechanism by which most of the bacteriocins from LAB exert their antibacterial effect (Ghrairi et al., 2012). In class II, this mechanism is triggered when bacteriocin binds to a protein-receptor on the cell membrane of the target bacteria, although some authors believe that such protein-receptor does not appear to be essential for binding (Chen, Shapira, Eisenstein, & Montville, 1997; Jack, Tagg, & Ray, 1995).

Although it has been reported that bacteriocins show a bactericidal mode of action centered against homologous species, some of them have similar activity against food-borne pathogens; including the bacteriocin produced by *P. acidilactici* M that inhibits a large number of bacteria, such as *S. aureus*, *L. monocytogenes*, *C. perfringens*, *Bacillus coagulans*, *B. cereus*, and *A. hydrophila*; or the bacteriocin sakacin C2 produced by *L. sakei* C2a that inhibits many Gram-positive and Gram-negative bacteria (Hu, Zhao, Zhang, Yu, & Lu, 2013).

Nevertheless, the antimicrobial activity of bacteriocins is unstable and inconsistent, as it depends on the chemical and physical conditions of foods. There are factors that can interfere on the bacteriocin production by LAB such as unsuitable process conditions (pH, temperature, nutrients, among others), spontaneous cell loss on producing bacteriocin, infection of the cell by phage and the presence of competitive microorganisms in the medium (Schillinger, Geisen, & Holzapfel, 1996). Besides, the bacteriocin effectiveness would also be affected by the presence of bacteriocin-resistant microorganisms, enzymes (like proteases), occurrence of oxidation-reduction reactions, interaction with components of the food formula (fats, proteins, preservatives, pH, for instance) and diffusion restraints due to high salt concentration. Additionally, it can be influenced by the presence of nitrate and nitrite and low water activity, which can lead to inadequate distribution of the bacteriocin throughout the food product (Alves, Martinez, Lavrador, & De Martinis, 2006; Schillinger et al., 1996).

According to Kristo, Koutsoumanis, & Biliaderis (2008), bacteriocins can present a higher effectiveness when added into films and not directly incorporated to the product. In

fact, the production of bacteriocin by *L. plantarum* was higher in cellulose derivative films when compared with protein films (Sánchez-González, Saavedra, & Chiralt, 2013).

The mode of action of plantaricin C, a bacteriocin produced by strains of *L. plantarum*, depends on the target microorganism, showing a different behavior against Gram-positive bacteria (González et al., 1994). Thus, this bacteriocin showed a bactericidal mode of action, with the absence of concomitant or subsequent cell lysis, after being added to *L. sakei* CECT 906, reducing 50% the viability of exponentially growing cultures after 1 h without absorbance reduction. Meanwhile, it was observed that a bacteriolytic mode of action against *Lactobacillus fermentum* LMB 13554 decreases the viability of culture to 0.6% in only 5 min, accompanied by a drastic lowering in the optical density. Additionally, it showed complete lysis against *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 13551 and almost 100% viability reduction without an apparent decrease in optical density using *Lactobacillus helveticus* LMG 13555 and *Leuconostoc mesenteroides subsp.cremoris* NCDO 543. According to these authors, the lytic effect of plantaricin C was not observed with most LAB bacteriocins being potentially useful in accelerated food processing, releasing the enzymes contained in the starters into their substrates.

Other plantaricins show a broader antimicrobial spectrum inhibiting food-borne pathogens in addition to the closely related *Lactobacilli* species. For example, partially purified plantaricin 163, produced by *L. plantarum* 163 isolated from traditional Chinese fermented vegetables, inhibits Gram-positive bacteria (*S. aureus, L. monocytogenes, Bacillus pumilus, B. cereus, Micrococcus luteus*, *Lactobacillus thermophilus*, and *Lactobacillus rhamnosus*) and Gram-negative bacteria (*E. coli*, *P. aeruginosa*, and *Pseudomonas fluorescens*). However, it is unable to show antimicrobial activity against fungi such as *Penicillium notatum*, *Aspergillus niger*, and *Rhisopus nigricans* (Hu et al., 2013). Plantaricin LP84 showed activity against Gram-positive, Gram-negative, food-borne pathogenic, and spoilage bacteria (Suma, Misra, & Varadaraj, 1998). Plantaricin UG1 inhibits strains of the genera *Lactobacillus* and *Lactococcus*, in addition to food-borne pathogens such as *L. monocytogenes*, *B. cereus*, *C. perfringens*, and *Clostridium sporogenes* (Enan et al., 1996). Meanwhile, plantaricin MG presented inhibitory activity against Gram-positive and Gram-negative bacteria including *L. monocytogenes*, *S.aureus, Salmonella typhimurium*, and *E. coli* (Gong, Meng, & Wang, 2010).

4. *L. plantarum*

L. plantarum is one of the most widespread species of the genus *Lactobacillus* and is being widely used in food-related technologies (Brinques, Peralba, & Ayub, 2010; Sauvageau et al., 2012). This microorganism is a facultative heterofermentative LAB (Group II) (Bove et al., 2012a; Siezen & van Hylckama Vlieg, 2011). It is acid tolerant and considered as a safe microorganism (*Generally Regard as Safe* – GRAS) (Brinques et al., 2010). As it is a heterogeneous species, it is closely associated with the species *Lactobacillus pentosus*, *Lactobacillus paraplantarum* and, more recently, *Lactobacillus fabifermentans* (Parente et al., 2010; Siezen & van Hylckama Vlieg, 2011). This relationship was identified when more than 99% of their rRNA presented identical sequences, suggesting high phenotypic and genotypic similarity between species (Parente et al., 2010).

L. plantarum is the most important and versatile species of the group, and can be found as part of the microbiota of starchy foods and cereals, meats, dairy products, vegetables, fruits and drinks (Ricciardi et al., 2012). According to Todorov et al. (2011), different strains have been isolated from various niches, such as fermented milk, cheese, fermented cucumber, fermented olives, pasta, pineapple, grapefruit juice, sorghum beer and barley, molasses, boza, kefir and amasi. These strains have proven to be able to survive gastric transit and colonize the intestinal tract of humans and other mammals, and are considered as a member of the natural microbiota of these niches (Kleerebezem et al., 2003; Mathara et al., 2008). Some authors report that *L. plantarum* can adapt to various niches due to its ability to ferment a wide range of carbohydrates (Brinques et al., 2010; Prins et al., 2010; Todorov, 2008).

Furthermore, *L. plantarum* has traditionally been used in starter cultures in food fermentations (Bove et al., 2012b) and also as an ingredient for probiotic foods, such as the *L. plantarum* 299v strain, which is widely marketed (Siezen & van Hylckama Vlieg, 2011).

4.1. Bioproduction of bacteriocins from *L. plantarum*

Several studies have been focused on the optimization of culture medium and growth conditions in order to increase the production of bacteriocins by *L. plantarum*. There are many works describing the production of bacteriocins by strains of *L. plantarum*, namely: *L. plantarum* ST194BZ (Todorov & Dicks, 2005b), *L. plantarum* ST13BR (Todorov & Dicks, 2005a), *L. plantarum* ST414BZ (Todorov & Dicks, 2006b), *L. plantarum* ST664BZ (Todorov

& Dicks, 2006b), *L. plantarum* ST23LD (Todorov & Dicks, 2006a), *L. plantarum* ST341LD (Todorov & Dicks, 2006a), *L. plantarum* AMA-K (Todorov, Nyati, Meincken, & Dicks, 2007), *L. plantarum* ST26MS, *L. plantarum* ST28MS and *L. plantarum* ST32 (Todorov, 2008; Todorov, Gotcheva, Dousset, Onno, & Ivanova, 2000; Todorov, Powell, Meincken, Witthuhn, & Dicks, 2007; Todorov, Van Reenen, & Dicks, 2007).

Despite the great number of studies carried out until this moment, there is little knowledge about the growth conditions required for optimal production of bacteriocins by *L. plantarum*, and an ideal fermentation process has not been established (Todorov, Van Reenen, & Dicks, 2004). It is known that cell growth of *Lactobacillus* is directly influenced by the conditions of pH, temperature, medium composition, and aeration rate, among other factors. Since LAB are quite demanding on nutritional requirements, a rich medium is extremely necessary for good growth (Brinques et al., 2010).

4.2. Bacteriocins produced by *L. plantarum*

A wide variety of bacteriocins produced by different *L. plantarum* strains have been isolated and described. Table 1.1 shows some examples of bacteriocins produced by *L. plantarum* isolated from various fermented products, their respective biochemical features and some available genetic information. The examples are detailed as follows:

Table 1.1. Some examples of bacteriocins produced by *Lactobacillus plantarum* isolated from varius ecological niches.

Isolation niche	Strain name	Bacteriocin produced	Biochemical features	Genetic information	Some pathogens inhibited	Reference
Meat	*Lactobacillus plantarum* UG1	plantaricin UG1	Single-peptide with molecular mass between 3.0 and 10.0 kDa	Chromosomally encoded	*Listeria monocytogenes, Bacillus cereus; Clostridium perfringens, Clostridium sporogenes*	Enan, Essawy, Uyttendaele, & Debevere,1996
	Lactobacillus plantarum 35d	plantaricin 35d	Single-peptide with molecular mass estimated to be 4.5 kDa	Information about genetic determinates not shown	*Staphylococcus aureus, Listeria monocytogenes*, *Aeromonas hydrophila*	Messi, Bondi, Sabia, Battini, & Manicardi, 2001
	Lactobacillus plantarum LT154	plantacin 154	Single-peptide peptide with molecular mass estimated to be 3.0 kDa or less	Plasmid-encoded	*Enterococcus faecalis, Bacillus sp., Staphylococcus sp. Salmonella typhimurium*	Kanatani & Oshimura, 1994
	Lactobacillus plantarum SA6	plantaricin SA6	Single-peptide peptide with molecular mass estimated to be 3.4 kDa	Information about genetic determinates not shown	*Lactobacillus plantarum, Lactobacillus brevis, Leuconostoc sp, Listeria grayi*	Rekhif, Atrih, & Lefebvre, 1995
	Lactobacillus plantarum ST202Ch ST216Ch	bas ST202Ch and bac ST216	Single-peptide with molecular mass estimated to be 3.5 and 10 kDa, respectively	Chromosomally encoded	*Enterococcus faecium, Escherichia coli, Listeria monocytogenes, Pseudomonas spp., Staphylococcus aures.*	Todorov, Ho, Vaz-Velho, & Dicks, 2010

Fish	*Lactobacillus plantarum* PMU 33	plantaricin W	Two-peptide (α and β-peptide) with molecular masses estimated to be 3.2 and 3.0 kDa, respectively	Chromosomally encoded	*Listeria monocytogenes, Bacillus cereus, Staphylococcus aures, Enterococcus faecium, Enterococcus faecalis.*	Noopakdee et al., 2009
	Lactobacillus plantarum BF001	plantaricin F	Single-peptide with molecular mass between 0.4 and 6.7 kDa	Information about genetic determinates not shown	*Staphylococus aures, Salmonela typhimurum, Listeria monocytogenes, P. aeruginosa.*	Fricourt, Barefoot, Testin, & Hayasaka,1994
Fruits and vegetables	*Lactobacillus plantarum* ST28MS and ST16MS	ST28MS and ST16MS	Single-peptide with molecular masses estimated to be 5.5 and 2.8 kDa, respectively	Chromosomally encoded	*Lactobacillus sakei, Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, Escherichia coli, Acinetobacter baumanii*	Todorov & Dicks, 2005a
	Lactobacillus plantarum C11	plantaricin EF, plantaricin JK and inductor factor plantaricin A	Two two-peptide with data not shown about molecular mass	Chromosomally encoded	*Lactobacillus sp.; Pediococcus sp.; Leuconostoc sp. And Streptococcus sp,*	Daeshel, McKenney, & McDonald, 1990
	Lactobacillus plantarum LPC010	plantaricin S and plantaricin T	Two-peptide, where plantaricin S had molecular mass about 2.5 kDa and the molecular mass of plantaricin T was not detected	Possible chromosomally encoded	*Propionibacterium sp., Clostridium tyrobutyricum, Enterococcus faecalis*	Díaz, Sánchez, Desmazeaud, Barba, & Piard, 1993

	Lactobacillus plantarum ST16Pa	bacteriocin ST16Pa	Single-peptide peptide with molecular mass estimated to be 6.5 kDa	Information about genetic determinates not shown	*Enterococcus faecalis* *Enterococcus faecium,* *Listeria monocytogenes,* *Listeria innocua,* *Staphylococcus aureus,* *Streptococcus sp.,* *Pseudomonas sp.*	Todorov et al., 2011
	L. plantarum 163	plantaricin 163	Single-peptide with molecular mass about 3.5 kDa	Information about genetic determinates not shown	*Staphylococcus ureus, Listeria monocytogenes, Bacillus pumilus, Bacillus cereus,* *Micrococcus luteus, Lactobacillus thermophilus, Lactobacillus rhamnosus, Escherichia coli, Pseudomonas aeruginosa, Pseudomonas fluorescens*	Hu, Zhao, Zhang, Yu, & Lu, 2013
Milk products	*Lactobacillus plantarum* AMA-K	bacteriocin AMA-k	Single-peptide with molecular mass about 2.9 kDa	Information about genetic determinates not shown	*Enteroccus spp.* *Escherichia col,* *Klebsiella pneumoniae* *Listeria spp.*	Todorov, Nyati, Meincken, & Dicks, 2007a
	Lactobacillus plantarum WHE92	pediocin AcH	Single-peptide with molecular mass about 4.5 kDa	Plasmid-encoded	*Listeria monocytogenes*	Ennahar et al., 1996
	Lactobacillus plantarum LB-B1	pediocin LB-B1	Single-peptide with molecular mass estimated between 2.5 and 6.5 kDa	Plasmid-encoded	*Listeria,* *Lactobacillus, Streptococcus,* *Enterococcus, Pediococcu* and *Escherichia coli*	Xie et al., 2010

	Lactobacllus plantarum ST8KF	BacST8KF	Single-peptide with molecular mass about 3.5 kDa	Information about genetic determinates not shown	*Lactobacillus casei, Lactobacillus salivarus, Lactobacillus cuvatus* and *Listeria innocua*	Powell, Witthuhn, Todorov, & Dicks, 2007
Cereals	*Lactobacillus plantarum* ST13BR	bacteriocin ST13BR	Single-peptide with molecular mass about 10.0 kDa	Information about genetic determinates not shown	*P.aeruginosa; Enterococcus faecalis; Klebsiella pneumonia* and *Escherichia coli*	Todorov, Van Reenen, & Dicks, 2004b
	Lactobacillus plantarum ST194BZ	ST194BZ(α) and ST194BZ(β)	Two-peptide (α and β-peptide) with molecular mass estimated to be 3.3 and 14.0 kDa, respectively	Information about genetic determinates not shown	*Enterococcus faecalis; Escherichia coli; Enterobacter cloacae; P. aeruginosa*	Todorov & Dicks, 2005b
	Lactobacillus plantarum 423	plantaricin 423	Single-peptide with molecular mass about 3.5 kDa	Plasmid-encoded	*Bacillus cereus, Clostridium sporogenes, Enterococcus faecalis, Listeria spp*. and *Staphylococcus spp.*	Reenen, Dicks, & Chikindas, 1998

• Meat:

Various bacteriocinogenic strains of *L. plantarum* have been isolated from sausages obtained from different manufacturers under distinct ripening times (Garriga, Hugas, Aymerich, & Monfort, 1993).

Enan et al. (1996) isolated an antimicrobial substance produced from *L. plantarum* UG1 obtained from dry sausage. This substance was capable of inhibiting other strains of *Lactobacillus* and *Lactococcus* and some pathogenic strains, such as *L. monocytogenes*, *B. cereus*, *C. perfringens* and *C. sporogenes.* This antimicrobial compound was characterized as a bacteriocin and named plantaricin UG1. This is a single-peptide with a molecular mass between 3.0 and 10.0 kDa and its production appeared to be chromosomally encoded. In addition, a LAB isolated from Italian sausages produced a bacteriocin, called plantaricin 35d (4.5 kDa), having high antimicrobial activity against food pathogens (*S. aureus*, *L. monocytogenes* and *A. hydrophila*) (Messi et al., 2001).

The sausage has been employed as a continuous source of bacteriocinogenic cultures. For instance, Kanatani & Oshimura (1994) reported the production of a plasmid encoded bacteriocin called plantacin 154 with a molecular mass about 3.0 kDa or less, produced by *L. plantarum* LT154 strain isolated from dry sausage. Meanwhile Rekhif, Atrih, & Lefebvre (1995) obtained plantaricin SA6 from *L. plantarum* SA6, an isolate from fermented sausage. This is a single-peptide with molecular mass of about 3.4 kDa and information about genetic determinants was not mentioned.

A study by Todorov, Ho, Vaz-Velho, & Dicks (2010) characterized bacteriocins produced by the *L. plantarum* ST202Ch and ST216Ch strains isolated from beloura or chorizo, a traditional Portuguese product made of pork meat. The chromosomally encoded single-peptide bacteriocins were denominated by bacST202Ch and bacST216Ch with molecular masses estimated to be 3.5 and 10 kDa, respectively, and were able to inhibit the growth of various Gram-positive and Gram-negative microorganisms considered deteriorative of meat products. The genes encoding bacteriocin ST202Ch were found identical to that reported for the structural gene encoding pediocin PA-1 (*ped*A, *ped*B, *ped*C, and *ped*D).

• Fish:

Noonpakdee et al. (2009) isolated the *L. plantarum* PMU 33 strain from som-fak, a Thai product made of fish with low salt contents. The bacteriocin purified and characterized from the culture supernatant consisted of two peptides with the molecular masses of 3.2 and 3.0 kDa. The molecular mass of this two-peptide bacteriocin was nearly identical to that of two-peptide plantaricin W (Plw), which consists of two peptides Plwα and Plwβ. The genes

encoding these two peptides amplified by PCR with Plw gene specific primer showed identical sequences to Plwα and Plwβ. This bacteriocin was able to inhibit a large number of Gram-positive microorganisms considered pathogens and food spoilage microorganisms, such as *L. monocytogenes*, *B. cereus*, *E. faecalis* and *S. aureus*.

Fricourt, Barefoot, Testin, & Hayasaka (1994) isolated the *L. plantarum* BF001 strain from the flesh of processed and refrigerated catfish. This strain was able to produce an antimicrobial substance designated plantaricin F, a single-peptide with a molecular mass between 0.4 and 6.7 kDa. It was active against some bacteria of the genus *Lactobacillus*, *Lactococcus*, *Listeria*, *Micrococcus*, *Leuconostoc*, *Pediococcus*, *Staphylococcus*, *Streptococcus*, *Salmonella* and *Pseudomonas*.

• Fruits and vegetables:

Two bacteriocins, ST28MS and ST26MS, produced by different *L. plantarum* strains, were isolated from molasses and partially characterized. Both bacteriocins with molecular masses estimated to be 5.5 and 2.8 kDa, respectively, showed unusual antimicrobial activity against Gram-negative bacteria, including *P. aeruginosa*, *E. coli* and *A. baumanii*. No plasmids were recorded for strains ST28MS and ST26MS, suggesting that these bacteriocins are chromosomally encoded (Todorov & Dicks, 2005a).

L. plantarum C-11 isolated from fermented cucumbers (Daeschel, McKenney, & McDonald, 1990) produced bacteriocins such as plantaricin EF and plantaricin JK (Anderssen et al., 1998). Plantaricin A, which was previously incorrectly identified as the bacteriocin responsible for the antimicrobial activity of *L. plantarum* C-11, induces the production of the bacteriocins mentioned above (Meyer, Larsen, Sletten, Daeschel, & Nes, 1993).

Díaz et al. (1993) identified two bacteriocins produced by *L. plantarum* LPC010 isolated from fermented green olives. These antimicrobial substances were named plantaricin S (2.5 kDa), which is produced during the logarithmic growth phase, and plantaricin T (molecular mass not determined), produced when the microorganism reaches the stationary growth phase. The genetic determinants for plantaricin S and T production do not appear to be plasmid encoded.

Todorov et al. (2011) isolated the *L. plantarum* ST16Pa strain from papaya (*Carica papaya*). The bacteriocin produced by this strain, named ST16Pa (6.5 kDa), showed activity against different species of the genus *Enterobacter*, *Enterococcus*, *Lactobacillus*, *Pseudomonas*, *Streptococcus*, *Staphylococcus*, and some genres of *Listeria* spp. No information was given about genetic determinants.

Plantaricin 163 is a novel bacteriocin produced by *L. plantarum* 163 isolated from traditional Chinese fermented vegetables by Hu et al. (2013). This bacteriocin showed a broad-spectrum inhibitory activity not only against LAB but also against other Gram-positive and Gram-negative bacteria including *S. aureus*, *L. monocytogenes*, *B. pumilus*, *B. cereus*, *M. luteus*, *L. thermophilus*, *L. rhamnosus*, *E. coli*, *P. aeruginosa*, and *P. fluorescens*. The physicochemical studies of this bacteriocin (3.5 kDa) are in agreement with the characteristic features of antimicrobial peptides, thus indicating the potential value of plantaricin 163 as a biopreservative in the food industry (Hu et al., 2013). No information was given about genetic determinants.

Other bacteriocins are plantaricin C19 produced by *L. plantarum* C19 isolated from fermented cucumbers (Atrih, Rekhif, Moir, Lebrihi, & Lefebvre, 2001); plantaricin NA, produced by *L. plantarum* sp. isolated from vegetable origin (Olasupo, 1998); plantaricin-149, produced by *L. plantarum* NRIC 149 isolated from pineapple (Kato et al., 1994); and plantaricin D produced by *L. plantarum* BFE 905 isolated from "Waldorf" salad (Franz, Du Toit, Olasupo, Schillinger, & Holzapfel, 1998).

• Milk-based products:

Todorov, Nyati, Meincken, & Dicks (2007) reported the production of bacteriocin AMA-K by the *L. plantarum* AMA-K strain isolated from amasi, a product traditionally made of fermented milk that is consumed in different regions of Southern Africa, including Zimbabwe, South Africa and Lesotho. The bacteriocin AMA-K (2.9 kDa) inhibited the growth of *Enterococcus* spp., *E. coli*, *Klebsiella pneumoniae* and *Listeria* spp. No information was given about genetic determinants.

González, Arca, Mayo, & Suárez (1994) reported the production of plantaricin C by the *L. plantarum* LL441 strain isolated from Cabrales cheese. The bacteriocin showed bactericidal activity, followed by, in some cases, cell lysis.

Xie et al. (2011) demonstrated the presence of pediocin LB-B1 (single-peptide with molecular mass estimated between 2.5 and 6.5 kDa), which was produced by *L. plantarum* LB-B1 isolated from koumiss, a traditional Chinese fermented dairy product. In particular, this bacteriocin was active against *Listeria, Lactobacillus, Streptococcus, Enterococcus, Pediococcus* and *E. coli* strains. The genetic determinants for pediocin LB-B1 production appeared to be plasmid-encoded.

Powell, Witthuhn, Todorov, & Dicks (2007) isolated *L. plantarum* ST8KF from kefir, a carbonated refreshing drink, and its bacteriocin (single-peptide with molecular mass about

3.5 kDa) showed activity against different microorganisms including *L. casei*, *L. salivarius*, *Lactobacillus curvatus* and *L. innocua.* No information was given about genetic determinants.

Other bacteriocins include plantaricin MG (molecular mass about 2.1 kDa) produced by *L. plantarum* KLDS1.0391 and isolated from "Jiaoke", a traditional fermented cream from China by Gong et al. (2010), being active against Gram-positive and Gram-negative bacteria including *L. monocytogenes*, *S. aureus*, *S. typhimurium* and *E. coli* and plantaricin LC74 (single-peptide with molecular mass about 5 kDa), produced by *L. plantarum* LC74 isolated from crude goat's milk. This bacteriocin showed a narrow spectrum of activity against several strains of mesophilic lactobacilli including *L. plantarum*, *Lactobacillus brevis* and *Lactobacillus biichneri* (Rekhif, Atrih, & Lefebvre, 1994).

• Cereals:

Todorov, Van Reenen, & Dicks (2004) reported the production of the bacteriocin ST13BR (single-peptide with molecular mass about 10 kDa) by the *L. plantarum* ST13BR strain isolated from barley beer, a traditional drink made of fermented corn, barley, soy flour and sugar, produced in South Africa. This bacteriocin was effective against *L. casei*, *P. aeruginosa*, *E. faecalis*, *K. pneumoniae* and *E. coli.* No information was given about genetic determinants.

Todorov & Dicks (2005a) described the isolation of the *L. plantarum* ST194BZ strain from boza, a fermented beverage typically from the eastern Balkan countries. It is one of the most traditional drinks produced by the fermentation of different cereals with the use of yeast and LAB. The ST194BZ strain was able to produce two types of bacteriocins, termed ST194BZα (3.3 KDa) and ST194BZβ (14 kDa), being active against a broad range of pathogens and spoilage bacteria including *E. faecalis*, *E. coli*, *Enterobacter cloacae* and *P. aeruginosa.* No information was given about genetic determinants.

Reenen et al. (1998) isolated the *L. plantarum* 423 strain from sorghum beer. This strain produced the bacteriocin plantaricin 423 (single-peptide with molecular mass about 3.5 kDa), which is capable of inhibiting a wide range of microorganisms, such as *B. cereus*, *C. sporogenes*, *E. faecalis*, *Listeria* spp. and *Staphylococcus* spp. DNA hybridization studies have shown homology between the plasmid DNA of *L. plantarum* 423 and the pediocin PA-1 operon. This suggests that plantaricin 423 is plasmid-encoded.

Additionally, the production of other bacteriocins has been described, including plantaricin K, produced by *L. plantarum* DK9 isolated from "fufu", a fermented cassava product (Olukoya, Tichaczek, Butsch, Vogel, & Hammes, 1993); plantaricin ST31, produced by *L. plantarum* ST31 isolated from sourdough (Todorov et al., 1999) and plantaricin KW30, produced by *L. plantarum* strain KW30 isolated from fermented corn (Kelly, Asmundson, & Huang, 1996).

4.3. Applications of *L. plantarum* and its bacteriocins

A wide variety of benefits associated with the use of *L. plantarum* as a potential probiotic has been reported in literature (Ningegowda & Gurudutt, 2012; Zago et al., 2011). In general, these studies reported that this LAB has been used to enhance intestinal barrier function and improve symptoms of irritable bowel syndrome (Anderson, Cookson, MacNabb, Kelly, & Roy, 2010; Ducrotté, Sawant, & Jayanthi, 2012). According to Axling et al. (2012), the use of *L. plantarum* can affect gut microbiota, lipid metabolism and inflammation in high-fat fed mice. Another example of health benefits was reported by Nguyen, Kang, & Lee (2007). In this study, *L. plantarum* PH04 was effective in cholesterol-lowering activities. On the other hand, Gallego et al. (2011) reported that the consumption of probiotic *L. plantarum* improves the general health status in older people.

Several substances have been produced by different *L. plantarum* strains isolated from different food sources. These strains of bacteriocinogenic *L. plantarum* are naturally present in these products, contributing to the improvement of organoleptic characteristics and playing an essential role in their biopreservation (Todorov, 2009).

Biopreservation is a technique used to prolong food shelf life and improve safety through the use of protective microbiota and/or its antimicrobial peptides, such as bacteriocins (Schillinger et al., 1996). The application of bacteriocins can help to reduce the use of chemical preservatives and/or the intensity of heat treatment, as well as prevent other physical treatments, satisfying the demand for “fresh”, “ready-to-eat” foods with few preservatives (Todorov, 2009).

The bacteriocins produced by *L. plantarum* or even the strain itself are used in different situations. Penteado et al. (2007) conducted a study by inoculating bacteriocins from *L. plantarum* in silage of mombaça grass, with the purpose of improving the fermentation profile according to the values of pH, NH_3, lactic acid and acetic acid, favoring the development of LAB and lowering dry matter losses.

Campagnol, Fries, Terra, Santos, & Furtado (2007) produced a starter culture with a *L. plantarum* strain in the culture medium of porcine plasma and verified the feasibility of its application in salamis. As a result, salamis made with a *L. plantarum* starter culture promoted a higher microbiological safety to salamis, as well as a significant improvement of their flavor.

In the therapeutic field, the topical application of lactobacilli is receiving attention due to the prevention of superficial skin and burn wound infections. Brachkova et al. (2011) studied the properties of *L. plantarum* immobilized with calcium alginate films and

investigated the antibacterial activity of these films in a model burn wound in rats. The study showed a significant decrease in the number of colonies of *P. aeruginosa* (one of the most frequently isolated pathogens in chronic infections, which was used to test the activity of *L. plantarum*), suggesting that the immobilization of *L. plantarum* by calcium alginate films may be a possible intervention for the prevention of infections caused by burns. Also in this context, Ramos et al. (2012) investigated the effects of *L. plantarum* supernatants on pathogenic properties of *P. aeruginosa*, such as adhesion, viability, virulence factors, biofilm formation, and quorum sensing signal expression. In this study, *L. plantarum* supernatants were able to inhibit pathogenic properties of *P. aeruginosa* by a quorum quenching mechanism. Also, the antipathogenic properties, along with the immunomodulatory, tissue repair, and angiogenesis properties in the *L. plantarum* supernatants, make them an attractive option in infected chronic wound treatment.

O'Shea et al. (2012) reported the problem related to the odor of pig manure. Conventional dietary strategies to reduce this odor can be costly, prevent nutrient digestibility, and receive varying responses. Alternatively, the authors proposed the use of *L. plantarum* in the diet of pigs, with or without supplementation of inulin, in order to reduce the manure odor without compromising the nutrient digestibility. It was found that dietary supplementation of pigs with *L. plantarum*, with or without inulin, reduces the manure odor.

4.4. Purification of bacteriocins produced by *L. plantarum*

Different strategies for the extraction of bacteriocins produced by *L. plantarum* from cultivation broths, and further purification to final products have already been described in literature (Atrih et al., 2001; Gong et al., 2010; Hata, Tanaka, & Ohmomo, 2010; Müller, Carrasco, Tonarelli, & Simonetta, 2009; Smaoui et al., 2010; Todorov, Velho, & Gibbs, 2004; Zhu, Zhao, Sun, & Gu, 2014). Purification methods, including salinization, solvent extraction, ultrafiltration, adsorption-desorption, ion exchange chromatography and high-performance liquid chromatography (HPLC), are the most common techniques (Parada et al., 2007). Other alternative purification methods include salting-out, gel filtration, or reverse-phase high-performance liquid chromatography (RP-HPLC) (Hu et al., 2013).

Some purification strategies with the respective specific activity and purification folds are shown in Table 1.2. All of them led to high levels of purification indicating their high degree of effectiveness.

Table 1.2. Strategies of purification to obtain bacteriocins produced by *Lactobacillus plantarum.*

Bacteriocin	Purification steps	Specific activity (AU/mg)	Purification (fold)	References
Plantaricin ZJ008	Culture supernatant	14.9	1.0	Zhu et al. (2014)
	Macroporous resin column	37.5	2.5	
	Cation exchenge chromatography	369.9	24.8	
	Gel filtration	838.7	56.2	
	HPLC	8556.7	573.1	
Plantaricin from *L. plantarum* LP31	Culture supernatant	85.5	1	Müller et al. (2009)
	Sep-Pack cartridges (C18)	5959	69.6	
	Gel-filtration chromatography	5900	689.5	
	RP-HPLC	506000	5914,6	
Plantaricin C19	Culture supernatant	455	1	Atrih et al. (2001)
	Release of adsorbed bacteriocin from producing cells	17808	39.1	
	Reverse phase high-performance liquid chromatography (HPLC)	409600	900.2	
Plantaricin MG	Culture supernatant	0.37	1	Gong et al. (2010)
	Ammonium sulfate precipitation	5.35	14.0	
	Gel filtration	44.64	20.0	
	Reverse-phase chromatography	9333.33	25.2	
BacTN635	Culture supernatant	2083	1	Smaoui et al. (2010)
	Ammonium sulfate precipitation	9904	4.7	
	Centrifugal microconcentrators	14310	6.8	
	Gel filtration	146104	70.1	
	HPLC	197368	94.7	
Plantaricin ASM1	Culture supernatant	253	1	Hata et al. (2010)
	Ammonium sulfate precipitation	1850	7.3	
	Cation exchange chromatography	11900	47.0	
	Octyl-Sepharose CL-4B column	20700	81,8	
	HPLC	10700	42.3	

The literature related to the purification of bacteriocins is vast, encompassing several types of protocols. However, all protocols involve the use of the HPLC technique as the final purification step (Hata et al., 2010; Smaoui et al., 2010; Zhu et al., 2014). Some authors employed the RP-HPLC technique, a variant of HPLC in which a hydrophobic stationary phase is employed, favoring, of course, the elution of polar molecules (Atrih et al., 2001; Gong et al., 2010; Müller et al., 2009). It must be borne out that by using the RP-HPLC technique as the final step, less purification procedures are required, thus decreasing the overall cost of the purification process.

Recently, a new procedure was proposed for the purification of macromolecules, including bacteriocins, based on the liquid-liquid extraction for aqueous two-phase micellar systems (ATPMS). This method can be applied for extracting bacteriocins directly from the fermented medium, leading to a simplification on the overall purification protocol of bacteriocins (Jozala, Lopes, Novaes, Mazzola, & Pessoa-Junior, 2012; Liu, Nikas, & Blankschtein, 1996; Dutra-Molino et al., 2014).

5. Conclusion

Nowadays, consumers tend to seek fresh and natural products, avoiding processed products containing chemical additives. Thus, many researchers began to look for natural and effective preservatives. The use of bacteriocins seems to be a great alternative, as they present activity against a wide range of food-borne pathogens and spoilage microorganisms.

Several substances have been isolated from various *L. plantarum* strains found in different niches, such as meat, fish, dairy products, fermented vegetables, cereals and fruits. Their application can be performed successfully in food-related fermentations, ensuring not only the organoleptic characteristics, but also contributing to increase the shelf life and safety of the final product.

Although there are many studies on the production of bacteriocins by *L. plantarum*, until this moment, there are no reports in literature describing the existence of a bacteriocin from *L. plantarum*, such as nisin and pediocin, available in the market. This fact could be due to the lack of an efficient or a low-cost purification strategy, which allows the bacteriocin produced by this microorganism to be commercially available. Therefore, future studies might be directed at the development of efficient and low-cost purification protocol.

CHAPTER II[1]

Effect of inulin on growth and bacteriocin production of *Lactobacillus plantarum* in stationary and shaken cultures

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ABSTRACT

The prebiotic effect of inulin added to MRS medium on growth and bacteriocin production by *L. plantarum* ST16Pa was investigated in stationary cultures in anaerobic jars with medium containing 0.025% sodium thioglycolate or in flasks shaken at 100 rpm. In the presence of 1% inulin in anaerobic stationary cultures, this strain produced lactic acid at a level that was 36.5% higher than in the absence of the polysaccharide. In shaken cultures without inulin, cell count was 54% higher than in the stationary ones. Under stationary conditions in anaerobic jars, the addition of inulin increased the maximum specific growth rate from 0.37 to 0.49 h^{-1} and reduced the generation time from 1.85 h to 1.40 h. Consequently, the exponential phase was shortened from 12 to 9 h when the cells were grown in stationary cultures with the oxygen scavenger. Despite this effect of inulin on growth rate, stationary cultures without inulin displayed higher antimicrobial activity against *Listeria monocytogenes* L104 (3,200 AU/mL) than cultures with inulin (1,600 AU/mL); therefore, inulin behaved as a compound able to accelerate growth rather than to stimulate bacteriocin production. The results presented in this study are very promising, since *L. monocytogenes* is a well-known foodborne pathogenic microorganism. Moreover, *L. plantarum* ST16Pa has proven to be a potential producer of a natural food preservative at an industrial level.

Keywords: bacteriocin, inulin, *Lactobacillus plantarum*, probiotic, stationary culture, shaken culture, supplementation.

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1. Introduction

Consumers have become more concerned about the amount of chemical additives in their diets and are seeking "natural" or chemical preservative free foods. Lactic acid bacteria (LAB) are widely used in the food industry to provide preservative functions, because of their ability to produce various antibacterial compounds, including bacteriocins (De Vuyst & Leroy, 2007). Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria that are active against other bacteria, either belonging to the same species or even across genera (Martinez et al., 2013).

Lactobacillus plantarum a generally recognized as safe (GRAS) microorganism is a LAB that has been widely used food processing (Barrangou, Lahtinen, Ibrahim, & Ouwehand, 2012; De Vuyst & Leroy, 2007; Sabo, Vitolo, González & Oliveira, 2014). Several *Lactobacillus* spp. have well-documented probiotic properties and can grow either in the absence or in the presence of O_2, which makes them aerotolerant anaerobic microorganisms (Götz, Elstner, Sedewitz, & Lengfelder, 1980). *L. plantarum* is a versatile species as it is present in the microbiota of starchy foods, cereals, meat, dairy products, vegetables, fruits, beverages and intestinal tract of mammals (Kleerebezem et al., 2003; Todorov & Franco, 2010).

Inulin is a prebiotic fermentable soluble fiber that cannot be digested by the enzymes of the human gastrointestinal tract; therefore, it enters the large intestine serving as substrate for intestinal beneficial bacteria, promoting their growth and improving their performance (Roberfroid, 2007). The stimulatory effect of this biopolymer on the growth of probiotic microorganisms can be explained by the increased level of fructose released by its partial hydrolysis and its subsequent assimilation through the glycolytic pathway (Oliveira, Perego, Oliveira, & Converti, 2012). Since the production of bacteriocins is often associated with high cell concentrations (Parente & Ricciardi, 1999), supplementation of the culture media with additional carbon sources such as inulin might stimulate their production.

The present study aimed at investigating *L. plantarum* ST16Pa growth and bacteriocin production in shaken and stationary cultures with and without the addition of inulin to the MRS medium.

2. Materials and methods

2.1. Bacterial strains and cultivation

The *Lactobacillus plantarum* ST16Pa strain used in this study was isolated from papaya (*Carica papaya*) and characterized as a bacteriocin producer and potential probiotic (Todorov et al., 2011, 2012). The microorganisms used as indicators in preliminary screening tests on filtrate samples from cultures grown under different conditions (shaken or stationary culture, with or without 1% inulin) were *Listeria monocytogenes* (L101, L104, L106, L302, L409, L506, L607, L620, L711, L724) and *Enterococcus* sp. (13LC23, 13BA22, 11EN3, 11EN4, 11EN5, 1LB8) provided by the Department of Food, Faculty of Pharmaceutical Sciences, University of São Paulo (São Paulo, SP, Brazil), and the Department of Veterinary, Faculty of Veterinary Medicine, Federal University of Viçosa (Viçosa, MG, Brazil), respectively. Only one concentration of inulin (1%) was tested because a preliminary (unpublished) study, in which this prebiotic was tested at different concentrations (1, 2 and 4%), showed no statistically-significant difference among results. Additional screening tests were performed exclusively on samples from stationary cultures without inulin using *Listeria innocua* 2061, 2864, 2871, 2865, 2860, 2712, 2711, 2863, 2193, 2873 and *Lactobacillus sakei* ATCC 15521, all provided by FioCruz (Rio de Janeiro, RJ, Brazil). BHI or MRS broth (DIFCO, Detroit, MI, USA) was used for storage and cultivation of *Listeria* strains or of the other strains, respectively. All microorganisms were stored at -80 °C in the presence of 40 % (v/v) glycerol.

To cultivate the indicator strains, 100 µL of stock cultures were added to 5 mL of medium and incubated at 100 rpm, 30 ± 0.5 °C for 18 h.

To prepare the *L. plantarum* ST16Pa inoculum, 1 mL of stock culture was inoculated into Erlenmeyer flasks containing 100 mL of MRS broth. The flasks were placed on a rotatory shaker at 100 rpm, 30 ± 0.5 °C for 12 h.

Under shaken conditions, 10% (v/v) of inoculum was transferred to Erlenmeyer flasks containing 100 mL of MRS broth with or without 1% (w/v) inulin and incubated in a shaker at 100 rpm, 30 ± 0.5 °C for 48 h. For stationary conditions, 10% (v/v) of inoculum was transferred into flasks containing 9 mL of MRS broth with or without 1% (w/v) inulin and incubated in jar without agitation. A GasPak EZ Container System (BD Diagnostic Systems, Hunt Valley, MD, USA) was used inside the anaerobic jar, and 0.025 % sodium thioglycolate

(oxygen reducer) was added to the broth to ensure hypoxia. Cultures were grown in triplicate, and samples were collected every 3 h during the first 15 h of fermentation and then every 6 h.

2.2. Growth, pH and biochemical measurements

Growth of *L. plantarum* ST16Pa was measured by counting colony-forming units (CFU) using serial dilutions in saline (0.85% w/v). One hundred mL of each dilution were spread over the surface of plates containing MRS broth supplemented with 2% (w/w) agar. The plates were incubated for 48 h. Subsequently, the values of the colony counts were converted to decimal log.

The progressive acidification of culture medium was followed with a pH meter (pH300M Analyser, São Paulo, SP, Brazil).

All chemicals used in the experiments were of analytical reagent grade. High purity glucose and lactic acid (Sigma-Aldrich, St. Louis, MO, USA) were used at concentrations from 0.1 to 2.0 g/L as standard solutions to prepare the calibration curve. In particular, the calibration curve was prepared by diluting the stock solutions with ultrapure water obtained from a Milli-Q system (Millipore, Bedford, MA, USA). The concentrations of glucose and lactic acid were determined by High Performance Liquid Chromatography (HPLC). Briefly, each sample was centrifuged at 25,000 *g* for 10 min using a microcentrifuge (U-32R, Boeckel, Hamburg, Germany). The supernatant was filtered through a membrane with 0.22 μm pore diameter (Millipore, Bedford, MA, USA) and then injected into an HPLC system (Shimadzu, model Prominence, Kyoto, Japan), equipped with the following modules: Two pumps (LC 20AD), column oven (CTO 20AC), degasser (model DGU-20A), an auto injector SIL-20ACHT adjusted to 30 μL volume injection, UV-vis SPD 20A, diode array SPDM 20A, with a working wavelength range of 190-230 nm, and refractive index (RID-10A) detectors, CBM controller 20A and Lab-Solutions software version 3.60. For the stationary phase, the Aminex HPX-87H ion exclusion column (300 x 7.8 mm, Bio-Rad Laboratories, USA) was used. Analyses were carried out at 50 °C, using 5.0 mM sulfuric acid as mobile phase at a flow rate of 0.6 mL/min and a wavelength of 215 nm was selected for the quantification.

2.3. Bacteriocin bioassays

Culture samples were centrifuged at 20,000 *g* at 4 °C for 10 min. The pH of the supernatants was adjusted to 6.0-6.5 by addition of 1.0 M NaOH and heated to 80 °C for 10

min to inactivate proteases. Bacteriocin activity tests were performed according to the agar diffusion assay in Petri dishes containing 15 mL of BHI broth supplemented with 1% (w/w) agar and inoculated with an appropriate test microorganism. After solidification, 10 µL of cell-free supernatant were deposited over the plates, which were then incubated at 30 °C. Inhibition zones became visible after 18 to 24 h. Bacteriocin activity was quantified in triplicate by serial dilution of cell-free supernatant in 25 mM phosphate buffer at pH 6.5. The amount of bacteriocin produced by *L. plantarum* ST16Pa was expressed in arbitrary units per mL (AU/mL) by the equation AU/mL = D^n x 1000/P, where D = dilution factor, n = first dilution not exhibiting any inhibition zone, and P = volume of supernatant deposited onto the agar surface.

2.4. Statistical analysis

Biomass, pH, bacteriocin activity, glucose and lactic acid concentrations were submitted to analysis of variance (ANOVA) by the Statistica Software 12 (Tulsa, OK, USA).

3. Results and discussion

3.1. Acidification performance and cell growth kinetics

As illustrated in Figure 2.1, *Lactobacillus plantarum* ST16Pa was able to grow either in shaken or stationary culture. Both modes of cultivations exhibited very similar acidification performances mainly associated with the release of lactic acid, with initial pH around 6.4-6.6 and final values of 4.1 in both cases. *L. plantarum* ST16Pa stopped growing after approximately 24 h of cultivation, and the pH remained almost constant over the next 24 h. At this time, glucose was almost completely consumed. As suggested by Kouakou *et al.* (2010), such a low pH along with the presence of bacteriocin should be sufficient to prevent growth of foodborne pathogenic microorganisms such as *Listeria monocytogenes.*

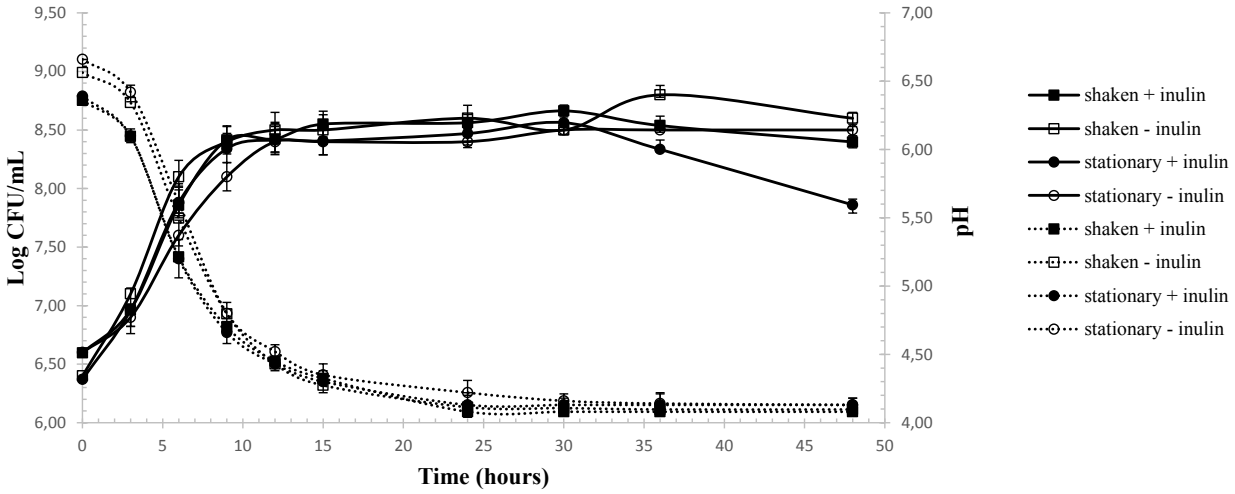

Figure 2.1. Growth curves of *L. plantarum* ST16Pa (continuous line) and pH values (pointed line).

The maximum cell concentration (X = 5.9 x 10^8 CFU/mL) in the shaken culture was more than 54% higher than that in the stationary one, the exponential growth phase was faster (9 h instead of 12 h) and the specific growth rate significantly higher (μ_{max} = 0.53 h^{-1} instead of 0.37 h^{-1}); consequently, the generation time was proportionally lower (Tg = 1.29 h vs. 1.85 h). These results show that the conditions adopted in shaken culture improved cell proliferation. Similar results were obtained by Gupta *et al.* (2011). Presumably, the agitation improved the distribution of nutrients, and the increased oxygen level in the medium could have led to higher yields of ATP per mol of consumed substrate (Fu & Mathews, 1999; Tortora et al., 2012).

The shaken culture exhibited a slightly higher cell count (X = 5.9 x 10^8 CFU/mL) than the stationary one (X = 3.2 x 10^8 CFU/mL); therefore, 1% (w/w) inulin was added in the medium to stimulate growth. However, no statistically-significant effect was observed on this parameter (X = 5.3 x 10^8 and 3.5 x 10^8 CFU/mL for shaken and stationary cultures).

These results disagree with those of Huebner *et al*. (2007), who observed that supplementation of the culture medium with inulin led to a drastic decrease in cell concentration, depending on the strain of *L. plantarum*. Saulnier *et al.* (2007) claimed that inulin addition did not influence *L. plantarum* WCSF1 cell count, because the fructo-oligosaccharides released from inulin hydrolysis likely induced upregulation of genes involved in primary metabolism (fructokinases, phosphoenolpyruvate transport chain, β-fructofuranosidade, α-glucosidase) and regulated negatively genes for protein formation, cell wall and lipid production. A similar occurrence may have taken place with the strain employed in this study.

On the other hand, the values of μ_{max} and Tg were appreciably influenced by the addition of inulin. As expected, the highest value of μ_{max} (0.53 h^{-1}) and lowest value of Tg (1.29 h) were obtained in shaken culture, but in the presence of 1% (w/w) inulin the former parameter decreased to 0.48 h^{-1} and the latter increased by about 10%. Under stationary conditions, these variations were opposite and even more significant, in that μ_{max} increased from 0.37 to 0.49 h^{-1}, Tg decreased from 1.85 to 1.40 h, and consequently the exponential phase was shortened from 12 to 9 h. The values of cell concentration, μ_{max} and Tg of each culture condition were summarized in Table 2.1. Nagpal & Kaur (2011) obtained qualitatively similar results using various strains of *L. plantarum* under comparable conditions, even though no cell count was reported.

Table 2.1. Kinetic parameters of each culture conditions analyzed.

	Cultures Conditions			
	Shaken	**Shaken + 1% inulin**	**Stationary**	**Stationary + 1% inulin**
Cell concentration (UFC/mL)	5.9 x 10^8	5.3 x 10^8	3.2 x 10^8	3.5 x 10^8
μ_{max} (h^{-1})	0.53	0.48	0.37	0.49
Tg (h)	1.29	1.44	1.85	1.40

3.2. Glucose consumption and lactic acid release

As shown in Figure 2.2, both stationary and shaken cultures released great amounts of lactic acid, but when stationary cultures were supplemented with 1% (w/w) inulin, the polysaccharide stimulated the production of lactic acid, whose concentration was 36.5 % higher than that in the absence of inulin. Glucose concentration was about the same under all culture conditions (ca. 20 g/L); however, in the presence of inulin, it is likely that some fructose was available in the culture medium as a result of partial hydrolysis of the polysaccharide. In this case, presumably, fructose monomers released from inulin, were assimilated by the EMP pathway, leading to higher concentrations of lactic acid under stationary conditions. A similar behavior was observed with *L. plantarum* WCFS1, which produced higher lactic acid concentrations in anaerobic culture when supplemented with oligofructose from inulin hydrolysis (Saulnier et al., 2007).

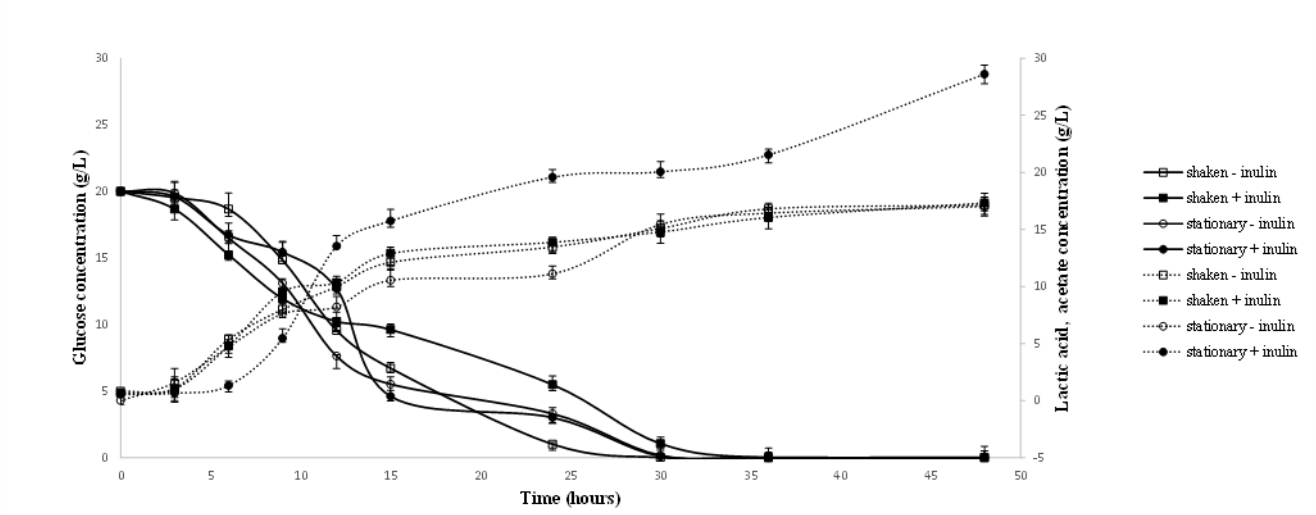

Figure 2.2. Concentrations of glucose (continuous line) and lactic acid (pointed line) during cultivations of *L. plantarum* ST16Pa.

3.3. Bacteriocin activity

Of the 16 indicator strains used in preliminary screening tests carried out under different conditions (shaken or stationary culture, with or without 1% w/w inulin), only three showed significant inhibition zones induced by the released bacteriocin, namely *L. monocytogenes* L101, L104 and L711 (See Figure 2.3 as an example).

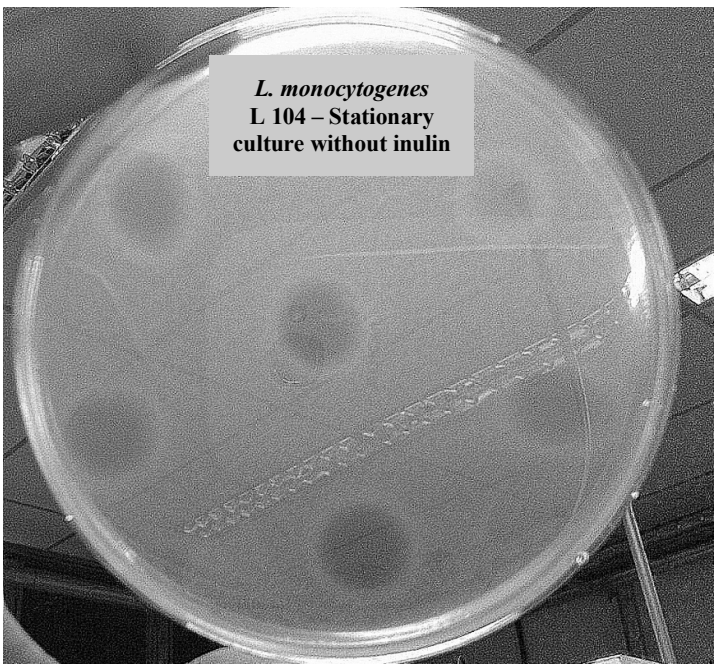

Figure 2.3. *In vitro* test of bacteriocin activity showing inhibition zones against *L. monocytogenes* L104.

The results of these tests, which are illustrated in Figure 2.4 in terms of mean inhibition zone versus time, show that the stationary culture without inulin provided the highest bacteriocin production and that the bacteriocin displayed a relatively narrow inhibition spectrum.

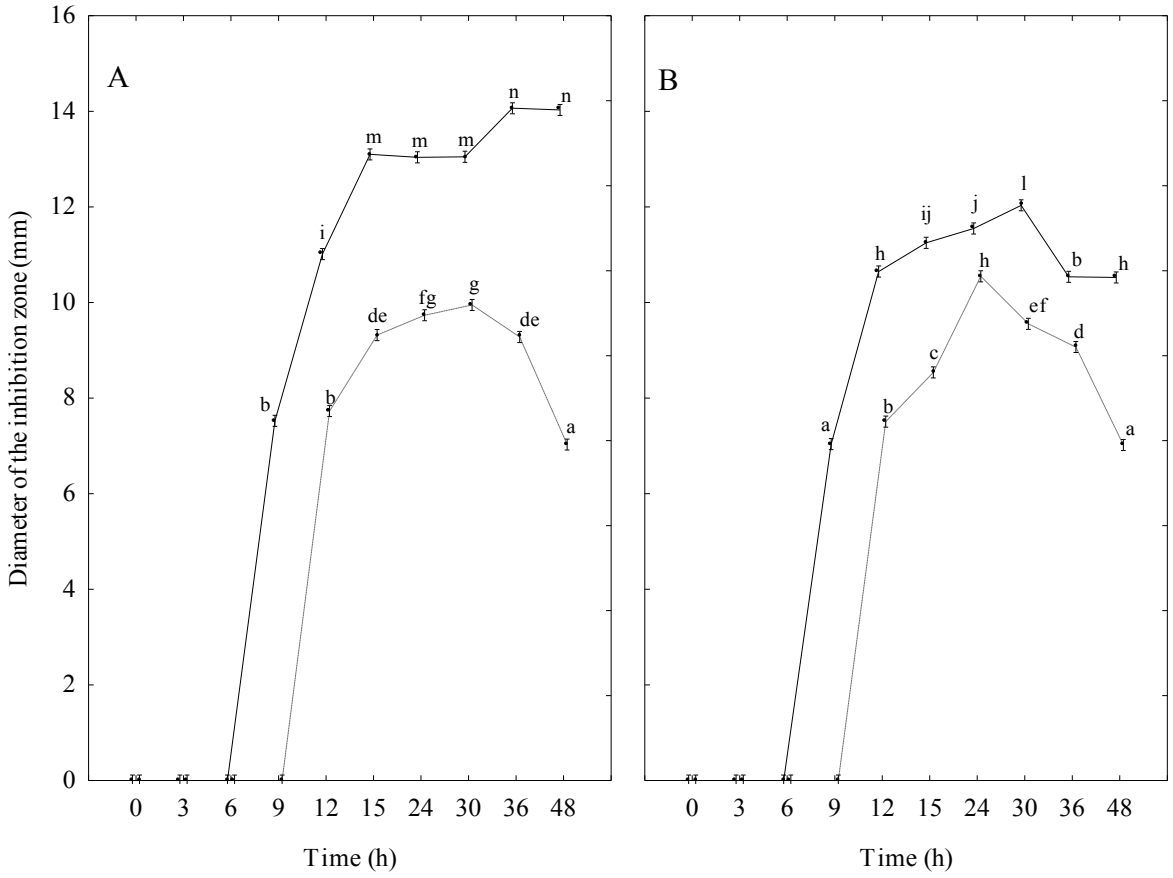

Figure 2.4. Inhibition zones against *L. monocytogenes* L104 either in shaken (dotted line) or stationary (continuous line) cultures, without (A) or with (B) 1% (w/w) inulin. Different letters mean statistically-significant differences according to the test of Tukey ($\alpha < 0.05$).

Therefore, 12 additional target microorganisms were tested exclusively with culture supernatants from stationary culture without inulin. The results of these tests, summarized in Table 2.2 together with those of preliminary tests, show that *Listeria innocua* 2052, 2873, 2711 and *Lactobacillus sakei* ATCC 15521 were sensitive to the bacteriocin and suggest that its action spectrum may be wider than evidenced by preliminary experiments. In fact, 25 % of the target microorganisms were shown to be sensitive to the bacteriocin, compared with 19 % in the preliminary experiments.

Table 2.2. Results of bacteriocin assay against differen target microorganism either in stationary or shaken flasks, with or without 1% (w/w) inulin.

Indicator Strains	Cultures Conditions			
	Shaken	Shaken + 1% inulin	Stationary	Stationary + 1% inulin
L. monocytogenes 106	-	-	-	-
*L. monocytogene*s 620	-	-	-	-
L. monocytogenes 409	-	-	-	-
L. monocytogenes 104	+	+	+++	++
*L. monocytogene*s 607	-	-	-	-
L. monocytogenes 302	-	-	-	-
L. monocytogenes 101	+	+	+++	++
*L. monocytogene*s 724	-	-	-	-
L. monocytogenes 711	+	+	+++	++
L. monocytogenes 506	-	-	-	-
Enterococcus sp. 13LC23	-	-	-	-
Enterococcus sp. 13BA22	-	-	-	-
Enterococcus sp. 11EN3	-	-	-	-
Enterococcus sp. 11EN4	-	-	-	-
Enterococcus sp. 11EN5	-	-	-	-
Enterococcus sp. 1LB8	-	-	-	-
L. innocua 2061	NT*	NT*	-	NT*
L. innocua 2864	NT*	NT*	-	NT*
L. innocua 2871	NT*	NT*	-	NT*
L. innocua 2865	NT*	NT*	-	NT*
L. innocua 2860	NT*	NT*	-	NT*
L. innocua 2712	NT*	NT*	-	NT*
L. innocua 2711	NT*	NT*	+++	NT*
L. innocua 2863	NT*	NT*	-	NT*
L. innocua 2193	NT*	NT*	-	NT*
L. innocua 2873	NT*	NT*	+++	NT*
L. innocua 2052	NT*	NT*	+++	NT*
L. sakei ATCC 15521	NT*	NT*	+++	NT*

Legend: 0 - 2 mm (-); 2 - 9 mm (+); 9 - 11 mm (++); 11 - 14 mm (+++).

*NT - Not tested

The supernatant from stationary cultures without inulin showed the largest inhibition zones, corresponding to a bacteriocin activity of 1,600 AU/mL against *L. monocytogenes* L101 and L711, *L. innocua* 2873, 2711 and 2052, and *L. sakei* ATCC 15521, as well as an activity of 3,200 AU/mL against *L. monocytogenes* L104. However, when the last culture was supplemented with 1% (w/w) inulin, it showed a considerably smaller diameter of the inhibition zone and a reduction in the bacteriocin activity against *L. monocytogenes* L104 to 1,600 AU/mL (Table 2.3). Gomes *et al.* (2012) reported similar results for sakacin 1, in that

inulin reduced the bacteriocin activity from 6,400 to 3,200 AU/mL. Similarly, Todorov *et al.* (2011) observed that *L. plantarum* ST16Pa produced less bacteriocin when the culture medium was supplemented with fructose, i.e. the monosaccharide released from inulin hydrolysis. Moreover, Todorov & Dicks (2004) reported that bacteriocin production is strongly dependent on the type of nutrient source provided in the culture medium, and that its activity is not always related to cell mass or growth rate of the producer strain. In shaken flasks, the bacteriocin activity against the three preliminary sensitive indicator strains (*L. monocytogenes* L101, L104, L711) was 1,600 AU/mL regardless to the presence or the absence of inulin.

Table 2.3. Quantification of bacteriocin activity against the indicator strains.

Indicator Strains	Cultures Conditions			
	Shaken	**Shaken + 1% inulin**	**Stationary**	**Stationary + 1% inulin**
	Quantification of bacteriocin activity (AU/mL)			
L. monocytogenes 104	1600	1600	3200	1600
L. monocytogenes 101	1600	1600	1600	1600
L. monocytogenes 711	1600	1600	1600	1600
L. innocua 2711	*NT	*NT	1600	*NT
L. innocua 2873	*NT	*NT	1600	*NT
L. innocua 2052	*NT	*NT	1600	*NT
L. sakei ATCC 15521	*NT	*NT	1600	*NT

*NT – Not Tested

4. Conclusion

The results of shaken and stationary cultures of *Lactobacillus plantarum* ST16Pa in MRS medium either with or without supplementation of 1% (w/w) inulin were compared. *L. plantarum* ST16Pa was shown to effectively grow under both conditions, but the highest bacteriocin production (3,200 AU/mL) was obtained in stationary culture without inulin. Inulin, while accelerating cell growth, was not appreciably metabolized as a carbon source for bacteriocin production, thus behaving as an actual prebiotic ingredient. The stationary culture without inulin showed inhibition zones against various strains of *Listeria monocytogenes* (L101, L104, L711) and *Listeria innocua* (2052, 2873, 2711) as well as against *Lactobacillus sakei* (ATCC 15521), among which *L. monocytogenes* L104 resulted to be the most sensitive one. Taking into account that *L. monocytogenes* is a well-known foodborne pathogenic

microorganism, these results are quite promising for possible use of *L. plantarum* ST16Pa as a producer of a natural food preservative at an industrial level. However, further studies are needed to produce the bacteriocin in bioreactors, i.e. under more controlled conditions, as well as to check its actual effectiveness as a preservative in foods. In addition, because foods are often subject to processes inducing stress factors to bacteriocinogenic bacteria, it would be essential, from an industrial viewpoint, to identify what stress conditions could substantially enhance bacteriocin production by *L. plantarum* ST16P.

CHAPTER III[1]

Bacteriocin partitioning from the fermented broth of *Lactobacillus plantarum* ST16Pa in aqueous two-phase polymer systems composed of sodium sulfate and cholinium-based salts

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ABSTRACT

The partitioning of bacteriocin in a novel inexpensive and stable aqueous two-phase polymer system (ATPPS) composed by poly(ethylene glycol) (PEG) and sodium polyacrylate (NaPA) was studied. The ATPPS were formed by mixing both polymers with sodium sulfate (Na_2SO_4) or the ionic liquid choline chloride ([Ch]Cl) and a bacteriocin extract from the fermented broth of *Lactobacillus plantarum* ST16Pa. Bacteriocin showed high stability in different temperatures and pHs, as well as in the presence of all ATPPS components. For the partitioning of bacteriocin, the effect as hydrophobic and electrostatic interactions are the major driving forces for the bacteriocin partitioning. In this case, the partitioning studies show that bacteriocin partitions preferentially to the PEG-rich (top) phase ($K_{Bact} > 1$). However, the highest partition was achieved in polymeric based ATPPS using ionic liquids as electrolytes. The system with 8 wt% PEG 10,000/8 wt% NaPA/0.5 M [Ch]Cl presented the higher partition coefficient ($K_{Bact} = 32$), and this condition presented high selectivity since the most part of total proteins partitioned to NaPA-rich (bottom) phase ($K_P = 0.51$) achieving a selective of 62.75. As conclusion, polymeric based ATPPS using ionic liquids as electrolytes can be considered a new potential separation process; mainly as a first step for bacteriocin extraction from fermented broth by applying low polymer content and mild conditions.

Keywords: Bacteriocin; aqueous biphasic systems; poly(ethylene glycol); sodium polyacrylate; choline chloride ([Ch]Cl).

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1. Introduction

The wide use of chemical preservatives such as nitrites and sulfur dioxide to extend the shelf life of foodstuff may cause adverse effects on human health and also on the nutrition level of food (Rai, Pandit, Gaikwad, & Kövics, 2016). For this reason, consumers are increasingly worried about the amount of chemical additives present in their diets, which leads to a growing seek for natural or chemical-free foods (Boye & Arcand, 2013). This fact, coupled with the increasing demand for minimally processed foods with long shelf life and convenience, has attracted research interest in finding natural preservatives (Castro et al., 2011). In this sense, bacteriocins, most produced by lactic acid bacteria (LAB), can be a promising alternative to the preservatives available in the market (Giraffa, Chanishvili, & Widyastuti, 2010).

Bacteriocins are defined as bacterially produced, heat-stable peptides active against other bacteria than the producer (Cotter et al., 2005). Several purification protocols have been adopted to extract bacteriocin from LAB cultivation (Sabo et al, 2014). Generally, the first step involves the concentration of bacteriocin from the culture supernatant by ammonium sulfate precipitation (Yang, Johnson, & Ray, 1992). Since this method does not provide a high degree of purity, subsequent steps using preparative isoelectric focusing and/or multiple chromatographic techniques, including cation exchange, gel filtration, hydrophobic interaction, and reverse-phase liquid chromatography are necessary to achieve high purification levels of bacteriocins (Guyonnet, Fremaux, Cenatiempo, & Berjeaud, 2000). However, these methods have their own drawbacks, such as high operation costs, and usually protocols including several steps are employed resulting in low yields (Berjeaud & Cenatiempo, 2004).

In the search for alternative methods, liquid–liquid extractions carried out in various types of aqueous two-phase complex-fluid systems have been investigated for bioseparation needs (Mazzola et al., 2008). Aqueous two-phase polymers systems (ATPPS) can be applied for bacteriocins extraction directly from the fermented medium, leading to a simplification of the overall purification protocol (Dutra-Molino et al., 2014). Some studies demonstrated that ATPPS can be formed from polyethylene glycol (PEG) and sodium polyacrylate (NaPA) in the presence of salt, resulting in a PEG-enriched top-phase and a NaPA-enriched bottom phase (Pereira, Santos, Johansson, Teixeira, & Pessoa-Junior, 2012). The PEG/NaPA systems have some advantages such as low viscosity when compared to traditional polymer-polymer systems (PEG/dextran), easy of handling, rapid phase separation by gravity within a few minutes, relatively low-cost chemicals, and are environmentally friendly (Barros et al., 2014;

Johansson, Feitosa, & Pessoa-Junior, 2011). PEG is an uncharged polymer while NaPA is negatively charged, thus the entropic penalty of counter-ion compartmentalization leads to a minimal concentration of salt being required for the formation of two aqueous phases (Johansson et al., 2011).

In previous works PEG/NaPA based ATPPS have been used for the partitioning of different biomolecules, such as the green fluorescence protein (Johansson et al., 2008a), hemoglobin, lysozyme, glucose-6-phosphate dehydrogenase (Johansson, Magaldi, Feitosa, &, Pessoa-Junior 2008b), myoglobin, ovalbumin (Saravanan, Rao, Nair, & Ramasami, 2008), cytochrome c (Santos, Silva, Coutinho, Ventura, & Pessoa-Junior, 2015), proteases (Barros et al., 2014) and amyloglucosidade (Alcântara, Nascimento, Mourão, & Minim, 2013). More recently, the use of ionic liquids (ILs) have been proposed to improve ATPPS potential (Du, Yu, & Wang, 2007; Li, Liu, Pei, Wang, & He, 2012; Xu, Wang, Huang, Li, & Wen, 2015). ILs are salts, liquid at temperatures below 100°C, composed of large organic cations with dispersed charge and anions of either inorganic or organic nature (Freire et al., 2012). Gutowski et al. (2003) were the first to demonstrate the ability of ILs to induce phase separation in the presence of inorganic salts. Remarkably, IL-based ATPPS have been shown to overcome some of the main limitations of typical polymer-polymer or polymer-salt systems since it is possible to tailor the properties and affinities of the IL-rich phase by adequately choosing the IL chemical structure (Pereira, Rebelo, Rogers, Coutinho, & Freire, 2013). In general, IL-based ATPPS have shown a better extractive performance compared to traditional polymer based ATPPS (Freire et al., 2012)

Although few studies with bacteriocin partitioning/recovery using PEG/salts based ATPPS (Abbasiliasi et al., 2014; Lappe, Anna, & Brandelli, 2012; Li, Bai, Li, Cai, & Ouyang 2001; Li, Ouyang, & Bai, 2000; Sidek et al., 2016) and with aqueous two-phase micellar system (ATPMS) (Jozala et al., 2008; 2013) have already been evaluated, PEG/NaPA systems composed of salts or ILs have never been proposed before. In this context and based on the advantages above mentioned, our group investigated PEG/NaPA based ATPPS with the ionic liquid choline chloride ([Ch]Cl) to extract bacteriocin from fermented broth of *Lactobacillus plantarum* ST16Pa. Moreover, we evaluated PEG/NaPA based ATPPS with a neutral salt (Na_2SO_4) for comparison. A multifactorial experimental design was used to estimate the effects of pH and temperature on bacteriocin partitioning in ATPPS. The stability of bacteriocin in the presence of ATPPS components (PEG, NaPA and salts) was also investigated. To our knowledge this is the first study on the partitioning of the bacteriocin produced by *L. plantarum* ST16Pa in polymer-polymer systems.

2. Material and Methods

2.1 Materials

Polyethylene glycol (PEG) with molar mass 2,000, 6,000 and 10,000 g/mol, were purchased from Merck (Hohenbrunn, Germany). Polyacrylic acid (NaPA) 8,000 g/mol (45 wt%) and the salts, sodium sulfate (Na_2SO_4), and choline chloride ([Ch]Cl) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All solutions were prepared in sodium acetate 0.1 M buffer, pH 5.0, using water purified by a Millipore Milli-Q system (Bedford, MA, USA). The glassware used was washed in 50:50 ethanol: 1 M sodium hydroxide bath, followed by a 1 M nitric acid bath, rinsed copiously with Milli-Q water and, finally, dried in an oven at 70°C for 1 h. All other reagents were of analytical grade and used as received.

2.2 Microbial cultures and fermentation conditions

The microorganism used was the strain *Lactobacillus plantarum* ST16Pa, isolated by Todorov et al. [33] from a papaya species. As a microorganism indicative of bacteriocin antimicrobial activity, we used the *Listeria* strain *innocua* 6a CLIST 2860 (AL224/07), isolated from a dry-fermented sausage sample and provided by Collection of Listeria (CLIST) from Fundação Oswaldo Cruz (FioCruz, Rio de Janeiro, Brazil).

For bacteriocin production by *L. plantarum* ST16Pa, firstly, the strain was reactivated by adding 1 mL of cryopreserved strain stock into a 250 mL Erlenmeyer flask containing 100 mL of broth medium Man, Rogosa and Sharpe (MRS) (DIFCO, Detroit, MI, USA) and incubating at 30°C on a rotatory shaker at 100 rpm for 24 h. Subsequently, 10 vv% of this culture was used to inoculate 1 L Erlenmeyer flasks containing 500 mL of MRS broth, equally incubated at 30°C for 24 h.

To culture the bioindicator strain *L. innocua* 6a CLIST 2860, 10 mL of the broth medium Brain Heart Infusion (BHI) (DIFCO, Detroit, MI, USA) was inoculated with 1 mL of cryopreserved strain stock and kept overnight at 37°C on a rotatory shaker at 100 rpm.

2.3 Determination of bacteriocin antimicrobial activity

The 24 h culture broth of *L. plantarum* ST16Pa was centrifuged at 25,750x*g* for 15 min. The pH of the resulting cell-free supernatant (CFS) was adjusted to 6.0-6.5 with 1 M

NaOH to eliminate organic acids and heated to 80°C for 10 min to inactivate proteases. Finally, the CFS was filtered through a 0.22 μm membrane (Millipore, Bedford, MA, USA) and subsequently tested against the bioindicator strain *L. innocua* 6a CLIST 2860. The test was performed by the agar diffusion method in which the bioindicator culture broth was 100-fold diluted, which means approximately 10^7 Colony Forming Units per mL (CFU/mL). 1 mL of this dilution was transferred to a Petri dish (90 x 15 mm) containing 10 mL of melted BHI soft agar (containing 0.75 wv% of agar). Then, 20 μL of the CFS was spotted onto the agar surface. A period of 3 h was allowed for the diffusion of the supernatant at room temperature and after the plates were incubated at 30°C for 24 h. Subsequently, the inhibition halos were measured in four directions by a digital caliper gauge (Lee Tools, model 684132) and the antimicrobial activity of bacteriocin (A_{Bact}), defined in arbitrary units (AU), was calculated based on Sidek et al. (2016). The equation is represented as follows:

$$A_{Bact} = \left(\frac{\pi R^2}{V}\right) = AU/mL \qquad \text{(Eq. 1)}$$

where πR^2 are the inhibition (clear) zone area of the halo (in cm^2) and V is the volume (in mL) of CFS (sample) dropped onto the agar surface. Some results were presented as $\%A_{\text{Bact}}$, considering the initial bacteriocin activity (around 68.35 AU/mL) as 100%. All experiments were performed in triplicate and average values were presented.

2.4 Determination of total protein concentration

Total protein concentration was determined using the bicinchoninic acid method (BCA), compatible with polymers. Samples of 100 μL containing proteins and 2 mL of the BCA working reagent, prepared according to the manufacturer's instructions, were added to a test tube. After 30 min the optical density at 562 nm was determined in spectrophotometer using deionized water as blank. Absorbance values were correlated to protein concentration based on a calibration curve using bovine serum albumin (BSA) solutions from 0 to 1,000 μg/mL (equation obtained: y (ABS) = 0.0011x (μg/mL) + 0.0245, R^2 = 0.99).

2.5 Bacteriocin activity in different conditions

The effect of pH and temperature on bacteriocin activity after 1 h was studied using a 2^2 central composite design. Bacteriocin activity was defined as the residual antimicrobial activity after exposition to the pH and temperatures investigated; these values (AU/mL) were

correlated in terms of bacteriocin stability. A set of 12 experiments, which contained a factorial or fractional factorial matrix, with center points and star points to allow the estimation of the curvature, was performed. The range and levels of the components under study are given in Table 3.1. The solutions' pH were adjusted with 5 M HCl or 5 M NaOH. Subsequently, we evaluated the influence of ATPPS components, namely PEG 10,000 g/mol, NaPA 8,000 g/mol, Na_2SO_4 and [Ch]Cl, on the activity of bacteriocin after 1 and 24 h at 25°C. In these set of experiments different concentrations of compounds were investigated as follows: PEG 10,000 g/mol and NaPA 8,000 g/mol - 10 and 20 wt%; Na_2SO_4 and [Ch]Cl - 0.20, 0.35, 0.50 and 0.90 M.

Table 3.1. Factor levels of the 2^2 central composite design to study the bacteriocin of *L. plantarum* ST16Pa activity as function of independent variables pH and temperature.

Factors	Coded Levels				
	Axial (-1.414)	**Lower (-1)**	**Center (0)**	**Higher (+1)**	**Axial (+1.414)**
pH	3.2	4.0	6.0	8.0	8.8
Temperature (°C)	37.6	50	80	110	122.4

2.6 Bacteriocin partitioning in ATPPS

ATPPS were prepared in 15 mL graduated glass tubes by adding PEG, NaPA, salts (Na_2SO_4 or [Ch]Cl) in sodium acetate buffer (pH 5.0) with 150 μL of the sample containing bacteriocin (CFS) that was the last component added resulting in a 5 g total mass system. The system components were added by weighing and tubes were sealed with PARAFILM® and homogenized in an orbital shaker (Barnstead/Thermolyne, model 400110) at 8 rpm for 15 min at room temperature, in order to form a homogeneous single-phase solution. Then, the systems were kept in a thermo-regulated device for 1 h at 25°C, to reach partition equilibrium. Afterwards, the two coexisting polymer phases (top and bottom phases) were carefully collected using disposable syringes. The volume of each phase was measured and the sample analyzed for protein concentration and bacteriocin activity. Each measurement was performed in triplicate.

The influence of PEG molar mass, PEG concentration and NaPA concentration (independent variables) on the bacteriocin partition coefficient was investigated using 2^3 full factorial designs (11 experiments, Table 3.2). Two 2^3 full factorial designs were performed, one with Na_2SO_4 in the ATPPS and the other with [Ch]Cl, both salts in the concentration of 0.35 M. The molecular weight of NaPA was fixed in 8,000 g/mol. All systems were evaluated

in terms of the effect (interference) in halo inhibition and their values subtracted from the same system in the presence of the sample. These assays were considered as blank and performed in triplicate.

Table 3.2. Factor levels of the 2^3 full factorial design to study the partitioning of bacteriocin in ATPPS.

Factors	Coded levels		
	Lower (-1)	**Center (0)**	**Higher (+1)**
PEG (g/mol)	2,000	6,000	10,000
PEG (wt%)	8	12	16
NaPA 8,000 (wt%)	8	12	16

2.7 Partitioning parameters

The partitioning behavior of bacteriocin in ATPPS was quantified by the partition coefficient, K_{Bact}:

$$K_{Bact} = \frac{A_{top}}{A_{bot}} \quad \text{(Eq. 2)}$$

where A_{top} and A_{bot} are the bacteriocin activity in the top (PEG-rich) phase and bottom (NaPA-rich) phase, respectively. The activity balance ($\%AB_{\text{Bact}}$) was calculated according to Equation 3 and the recovery of bacteriocin in both phases ($\%REC_{top}$ and $\%REC_{bot}$) was calculated according to Equations 4 and 5:

$$\%AB_{Bact} = \left(\frac{A_{top}V_{top}+A_{bot}V_{bot}}{A_iV_i}\right)\times 100\% \quad \text{(Eq. 3)}$$

$$\%REC_{top} = \left(\frac{A_{top}V_{top}}{A_iV_i}\right)\times 100\% \quad \text{(Eq. 4)}$$

$$\%REC_{bot} = \left(\frac{A_{bot}V_{bot}}{A_iV_i}\right)\times 100\% \quad \text{(Eq. 5)}$$

where A_{i} is the bacteriocin activity in the stock solution added to the system, and V_{top}, V_{bot}, and V_{i} are the volumes of the top (PEG-rich) phase, bottom (NaPA-rich) phase and the bacteriocin stock solution initially added to the system, respectively.

The partition of bacteriocin (P_{Bact}) relative to the two systems (PEG/NaPA with Na_2SO_4 and [Ch]Cl) was quantified according to the equation:

$$P_{Bact} = \frac{K_{Bact\ PEG/NaPA/[Ch]Cl}}{K_{Bact\ PEG/NaPA/Na_2SO_4}} \quad \text{(Eq. 6)}$$

The partition coefficient of total proteins (K_{P}) was also evaluated according to Equation 7, as follows:

$$K_P = \frac{C_{top}}{C_{bot}} \quad \text{(Eq. 7)}$$

where C_{top} and C_{bot} are the total proteins concentration (μg/mL) in the top (PEG-rich) phase and bottom (NaPA-rich) phase, respectively.

2.8 Determination of electrical conductivity in ATTPS

The electrical conductivity of each phase of the ATPPS was determined in a conductivity device (METTLER TOLEDO, model MPC 227). Prior to measurements, the apparatus was calibrated by reading a standard solution of 12.88 mS/cm and air. Each phase was read in triplicate.

2.9 Statistical analysis

For the statistical analysis of both experimental designs presented above, the coded values of each of the independent variable were used. The 'Statistica' software, Version 7.0 (Statsoft, Tulsa, OK, USA) was used for data regression and graphical analysis. The statistical significance of the regression coefficients was determined by Fischer's test for analysis of variance (ANOVA) at a significance level $p \leq 0.05$. The coefficient of determination (R^2), which is a measure for fitting a generalized linear statistical model in relation to the observed values, was used to indicate how well the model could explain the observed values. To minimize the ANOVA error, the tests corresponding to the central point were replicated four times. The experimental and predicted values were compared to determine the validity of the models.

3. Results and Discussion

3.1 Effects of pH, temperature, and ATTPS components on bacteriocin activity

Since the main application of bacteriocin is as preservative in processed foods and these products are usually produced with temperature and pH variations (An et al., 2015), evaluation of bacteriocin stability in extreme conditions of pH and temperature is necessary. In this sense, the effects of pH (3.3 to 8.8), and temperature (37.6 to 122.4°C) on the bacteriocin activity after 1 h of incubation were determined employing statistical tools (Table 1). The choice of these values was based on the literature describing pH tolerance and thermoresistance of bacteriocins (Belgacem, Rehaiem, Bernárdez, Manai, & Castro, 2012),

and also in view of the possible application of bacteriocins as food preservatives regarding usual levels in their processing operations (for both variables).

As can be seen in the Pareto chart (Figure 3.1) the temperature both in the linear and quadratic level influenced the stability of bacteriocin. Regarding the pH, only the linear level showed a marginal effect. The Pareto chart shows an estimative effect of the variables and their interactions on the response variable. So, each bar length is proportional to the standardized effect and the vertical line shows the statistically significant variables ($p > 0.05$) (Mayerhoff, Roberto, & Franco, 2004). Our results show that low temperatures are better for bacteriocin stability and the lowest stability was observed at pH 6.0 and 122.4°C, showing that extreme conditions of heat lead to a loss of bacteriocin activity. The fitted surface presented in Figure 3.2 shows that bacteriocin is more stable at temperatures from 50 to 80°C in the range of pH from 3.0 to 8.0.

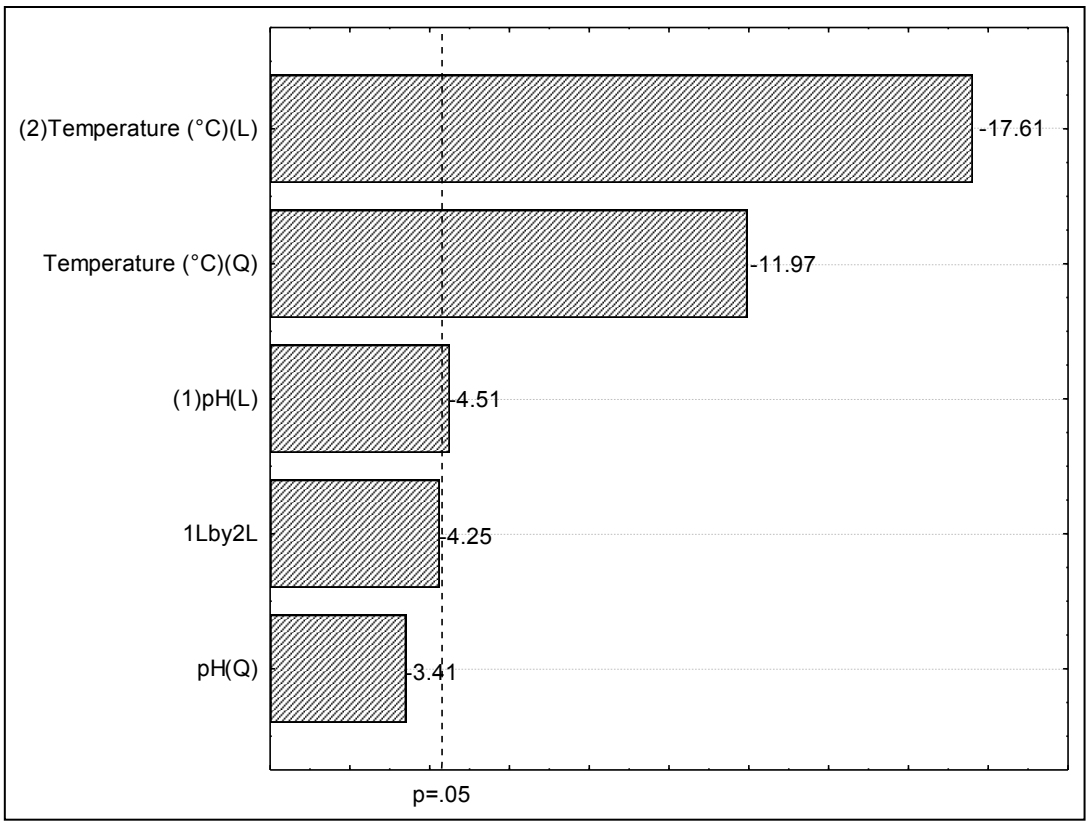


Figure 3.1. Pareto chart for the effects of pH (1) and temperature (2) on bacteriocin stability according to a 2^2 central composite design. The signal of the number in front of each bar corresponds to which magnitude of level could improve the variable response.

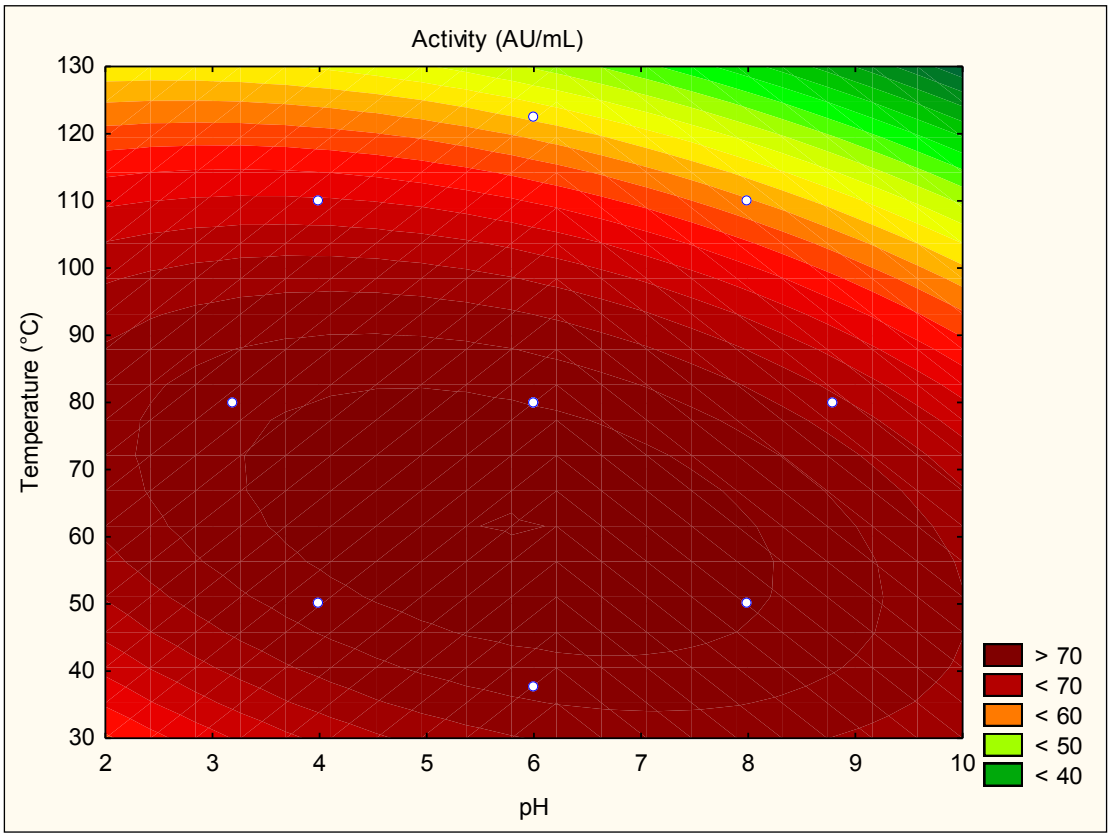


Figure 3.2. Fitted surface for the effects of pH and temperature on bacteriocin stability.

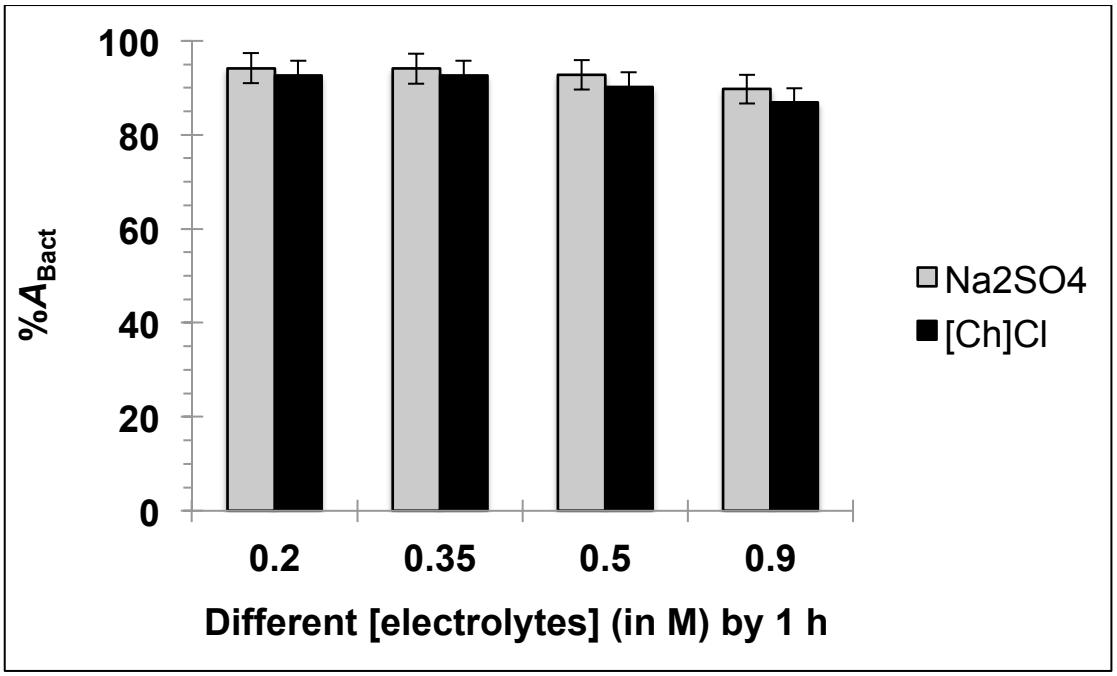
Some researchers have demonstrated that the overall initial bacteriocin activity was almost retained for temperatures up to 90°C, low pH (around 5.0) and short incubation times (around 10 min) (Belagacem et al., 2012). Guerra and Pastrana (2001) found similar results for the effect of pH/temperature on the stability of pediocin and nisin produced by *Pediococcus acidilactici* NRRLB5627 and *Lactococcus lactis subsp. lactis* CECT 539, respectively. Thermal stability at slightly acid media has been described for most bacteriocins (Guerra & Pastrana 2001; Todorov et al., 2011). According to the literature, the optimal pH range for bacteriocin production is around 4.5–5.5 (Belagacem et al., 2012; Calderón-Santoyo, Mendoza-García, García-Alvarado, & Escudero-Abarca, 2011). This behavior is attributed to their high content of glycine and to the formation, at a molecular level, of globular structures and strong hydrophobic interactions in the bacteriocin molecule (De Vuyt & Vandamme, 1994).

Our results also suggests that the bacteriocin evaluated here can tolerate the conditions normally encountered in food processing and are particularly interesting in medium acid fermented food products, including a number of fermented and ripened dairy and meat products.

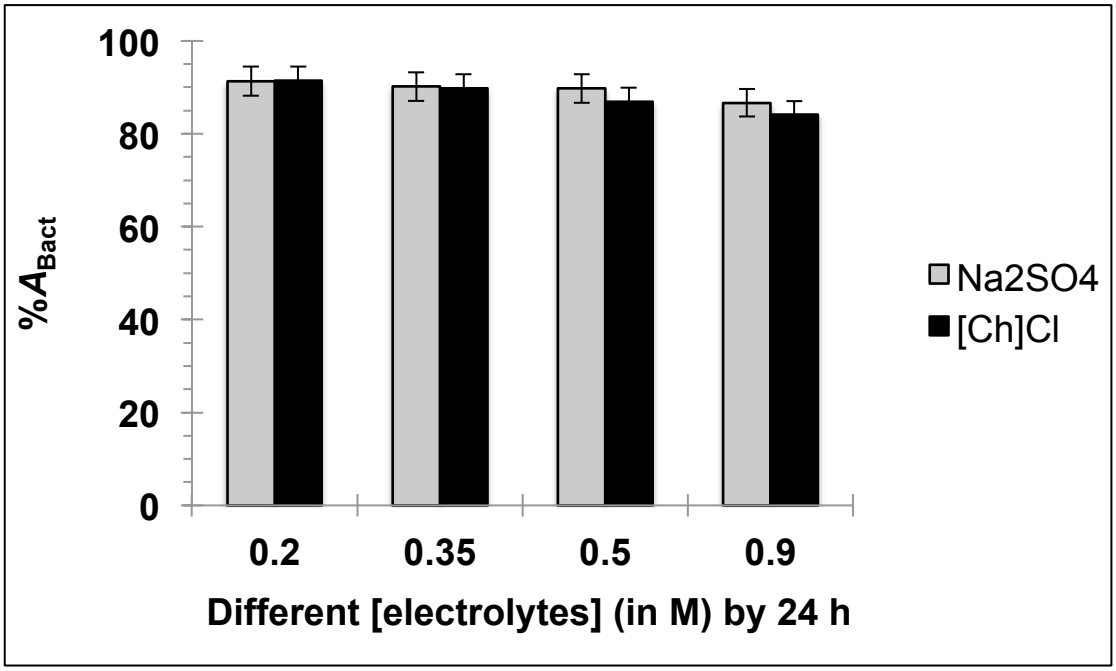
Additional studies were carried out to evaluate the bacteriocin stability in the presence

of the ATPPS components. We investigated the effect of the electrolytes Na_2SO_4 and [Ch]Cl at four different concentrations (0.2, 0.35, 0.5, and 0.9 M), for two different periods of time (1 h and 24 h), as can be seen in Figure 3.3. Based on the results with 1 h, the influence of both salts in the percentage of bacteriocin activity (%A_{Bact}) was relatively low under conditions evaluated. In the presence of Na_2SO_4, the %A_{Bact} varied between 95.1% (with 0.2 M) and 89.7% (with 0.9 M), respectively. In the case of [Ch]Cl the %A_{Bact} varied between 92.6 to 86.8% (at the same electrolytes concentration).

The same bacteriocin behavior can be observed after 24 h of exposition to the ATPPS components. The %A_{Bact} slightly decreases with the increase of electrolytes concentration. For Na_2SO_4 the %A_{Bact} values varied between 91.3% (with 0.2 M) to 86.6% (with 0.9 M). In the case of [Ch]Cl at the same electrolytes concentration, a variation in %A_{Bact} between 91.4 and 84.0% was observed. In general, the assays with 1 and 24 h showed similar results for the %A_{Bact} in the presence of both electrolytes, and only a small effect of activity loss occurs at high electrolyte concentrations (0.9 M).



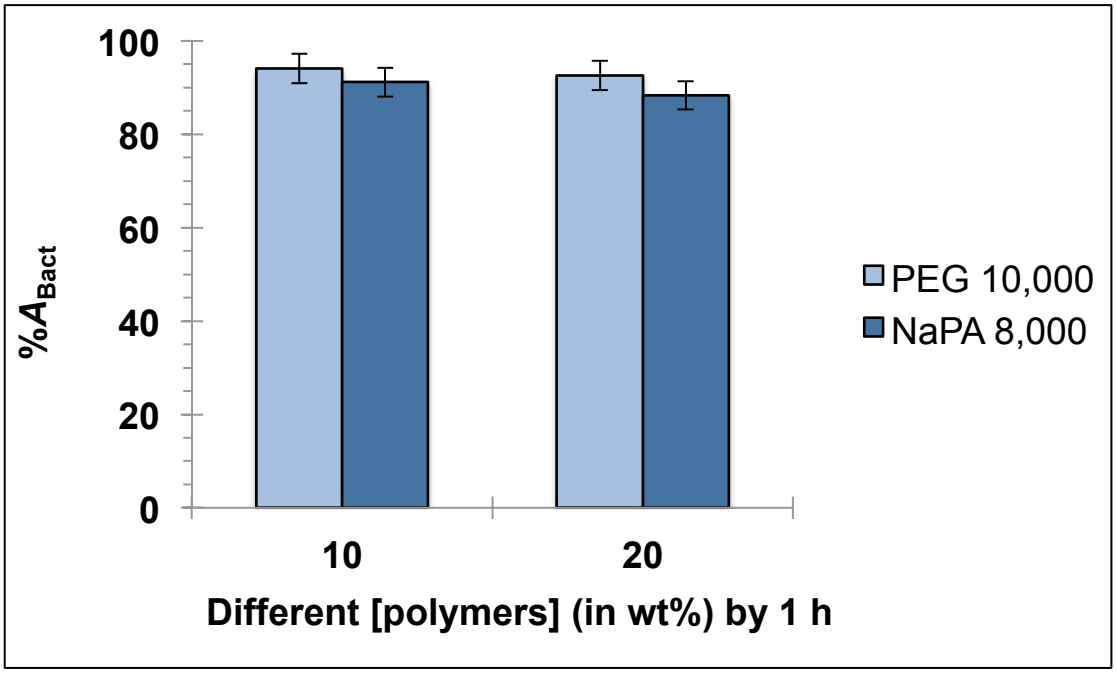
a)



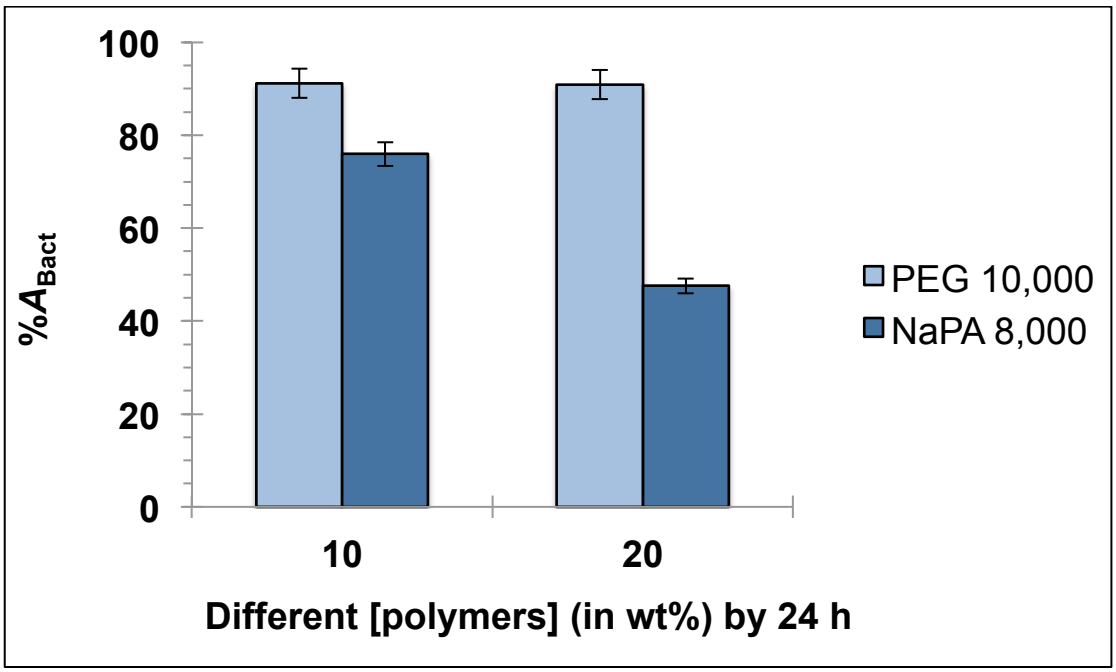
b)

Figure 3.3. Bacteriocin activity (%A_{Bact}) in the presence of different electrolytes (Na_2SO_4 and [Ch]Cl) concentrations: 0.2, 0.35, 0.5, and 0.9 M for 1 h (**a**) and 24 h (**b**). The error bars represent 95% confidence levels for the measurements.

The effect of polymers in the bacteriocin stability was also investigated (Figure 3.4). In this case, we evaluated the PEG with higher molecular weight used in our experiments (10,000 g/mol) and NaPA 8,000 g/mol, both at two different concentrations: 10 and 20 wt% (Fig. 4). Bacteriocin was found to be stable in the presence of PEG 10,000, with %A_{Bact} values above 91% even after 24 h of exposition. On the contrary, bacteriocin presented significant loss of activity in the presence of NaPA. The %A_{Bact} values were 91.1 (10 wt% NaPa) and 88.3 (20 wt% NaPa) after 1 h, and 76.0 (10 wt% NaPa) and 47.5% (10% NaPa) after 24 h. The NaPA polymer is strongly negatively charged (Johansson et al., 2008b) and can interact with oppositely-charged sites of biomolecules through electrostatic interactions, destabilizing or changing its molecular structure and causing loss of activity.



a)



b)

Figure 3.4. Bacteriocin activity (%A_{Bact}) in the presence of different polymers (PEG 10,000 g/mol and NaPA 8,000 g/mol) concentrations: 10 and 20 wt% by 1 h (**a**) and 24 h (**b**). The error bars represent 95% confidence levels for the measurements.

3.2 Bacteriocin partitioning in PEG/NaPA/Na_2SO_4 systems

An aqueous two-phase polymer system composed of PEG/NaPA and Na_2SO_4 at different conditions was investigated by a 2^3 full factorial design. This ATPPS is of particular interest because these polymers are nontoxic, biodegradable and approved by the Food and Drug Administration (FDA). Furthermore, they have been used to solubilize and stabilize pharmaceutical and biomedical products (Santos-Ebinuma, Lopes, Pessoa-Junior, Teixeira, 2015). Table 3.3 presents the experiments of the factorial design with the response-variable partition coefficient (K_{Bact}), as well as the experimentally obtained values for activity balance ($\%AB_{Bact}$) and bacteriocin recovery at both phases of the system ($\%REC_{top}$ and $\%REC_{bot}$).

Table 3.3. Results of the 2^3 full factorial design to study the partitioning parameters of bacteriocin in PEG/NaPA/Na_2SO_4 systems.

Experiments	K_{Bact}	$\%AB_{Bact}$	$\%REC_{top}$	$\%REC_{bot}$
16%PEG 2,000/ 16%NaPA	20.80	93	82	11
8%PEG 2,000/ 16%NaPA	13.42	98	81	17
8%PEG 2,000/ 8%NaPA	18.71	102	88	13
16%PEG 2,000/ 8%NaPA	11.48	68	44	24
***12%PEG 6,000/ 12%NaPA**	10.35 ± 0.37	81.9 ± 8.1	59.4 ± 7.2	22.5 ± 1.1
16%PEG 10,000/ 8%NaPA	18.34	45	32	14
16%PEG 10,000/ 16%NaPA	20.34	91	79	12
8%PEG 10,000/ 8%NaPA	21.34	72	61	11
8%PEG 10,000/ 16%NaPA	23.49	112	104	9

*The central point of full factorial design was repeated three times.

From the data presented in Table 3.3, bacteriocin partition coefficient was higher than one for all the conditions investigated, indicating that it preferentially partitions to the top (PEG-rich) phase of PEG/NaPA systems. The partition coefficient values varied between $10.35 < K_{Bact} < 23.49$. The Pareto chart (Figure 3.5) shows that the NaPA concentration is the main variable influencing K_{Bact} and lower PEG concentrations increase the partition of the target molecule, irrespectively of the PEG molecular weight. The highest K_{Bact} value was obtained with PEG 10,000 g/mol at the lower concentration (8 wt%) and higher concentration of NaPA (16 wt%).

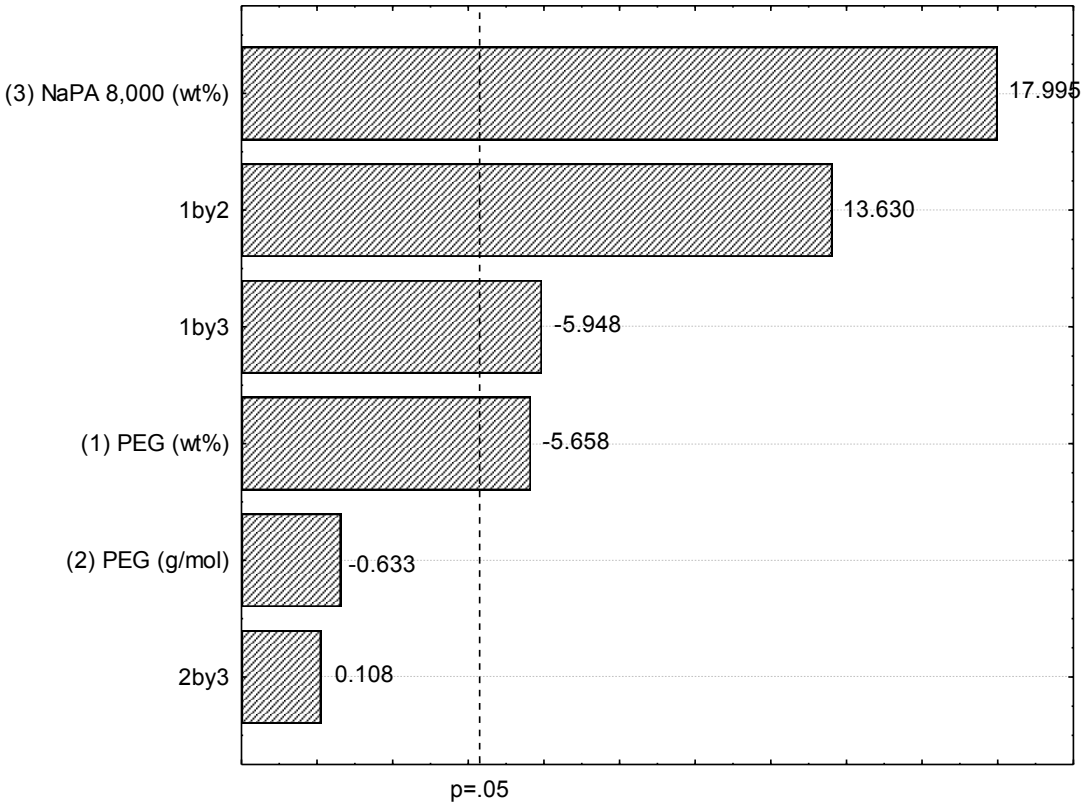


Figure 3.5. Pareto chart of ANOVA to identify the variables and interactions that exhibited significant effects on K_{Bact} in PEG/NaPA/Na_2SO_4 systems. The length of each bar is related to the standardized effect or interaction and the vertical line corresponds to the significant effect for a confidence interval of 95%.

Since NaPA is negatively charged, it can create repulsive or attractive interactions with the target biomolecule according to its charge. In this sense, previous work have demonstrated the NaPA influence on the extraction of biomolecules such as clavulanic acid in PEG/NaPA systems, which partitions preferentially to the PEG-rich phase due to electrostatic repulsions (Pereira et al., 2012). Additionally, previous studies with hemoglobin (Johansson et al., 2008b) and green fluorescent protein (Johansson et al., 2008a) showed that electrolytes

favor the partition of these negatively charged proteins to the PEG-rich phase; this behavior was more pronounced with Na_2SO_4 than NaCl. Since bacteriocin was highly excluded from the bottom phase (NaPA-rich), we believe it is negatively charged at pH 5.0 in which the ATPPS were performed. Similarly, pediocin PD-1 was also found to be negatively charged, with p*I* ~3.5 and molecular mass of ~3.5 kDa (Green, Dicks, Bruggeman, Vandamme, & Chikindas, 1997). In addition, the bacteriocin presented approximately the same size of pediocin PD-1 based on electrophoresis analysis (data not shown). Theses features support that the bacteriocin studied in this work can be classified as a pediocin-like bacteriocin, although studies on the amino acid sequence need to be performed to confirm our hypothesis (Cotter et al., 2005).

Based on in these considerations, the partitioning of the bacteriocin in ATPPS probably results from a combination of electrostatic repulsion by the negatively-charged bottom (NaPA-rich) phase and hydrophobic interactions with the polymeric top (PEG-rich) phase (with molecular weight of 10,000 g/mol the more hydrophobic PEG tested).

The activity balance (%AB_{Bact}) of the bacteriocin was close to 100% for all the conditions studied, except for 16 wt% PEG 10,000/8 wt% NaPA (%AB_{Bact} = 45%). Probably, at this condition the concentration of PEG 10,000 in the top phase after phase separation reaches values high enough to lead to a denaturing effect on the molecule. The recovery of the target molecule in the top (PEG-rich) phase (%REC_{top}) was higher than 60% for most of the conditions investigated, which confirms that the molecule preferentially partitioned into the top (PEG-rich) phase. The highest value of 104% was observed with 8 wt% PEG 10,000/16 wt% NaPA system. The %REC_{bot} as expected presented lower values, with values lower than 24% in the condition of the central point (12 wt% PEG 6,000/12 wt% NaPA system) of the 2^3 factorial designs.

Giving the promising results with PEG/NaPA/Na_2SO_4, we also evaluated a new PEG/NaPA system composed of cholinium-based salt in an attempt to increase the partitioning parameters of bacteriocin from fermented broth.

3.3 Bacteriocin partitioning in PEG/NaPA/[Ch]Cl systems

Table 3.4 shows that in general higher K_{Bact} were observed in PEG/NaPA systems in the presence of [Ch]Cl when compared with Na_2SO_4. The electrolyte nature affects bacteriocin partitioning to the PEG phase and, in this sense, the [Ch]Cl ionic liquids boost the partition towards the upper phase. Probably, the electrostatic forces involved in systems with ILs are more pronounced than in the presence of Na_2SO_4 (see the electrical conductivity

studies below).

Table 3.4. Factor levels used in the 2^3 full factorial designs to study the partitioning parameters of bacteriocin in PEG/NaPA/[Ch]Cl systems.

Assays	K_{Bact}	P_{Bact}	$\%AB_{Bact}$	$\%REC_{top}$	$\%REC_{bot}$
16%PEG 2,000/ 16%NaPA	26.90	1.29	99	94	5
8%PEG 2,000/ 16%NaPA	19.30	1.44	66	56	10
8%PEG 2,000/ 8%NaPA	29.09	1.55	82	76	5
16%PEG 2,000/ 8%NaPA	20.63	1.80	113	108	5
***12%PEG 6,000/ 12%NaPA**	9.27 ± 0.39	0.90 ± 0.05	99.5 ± 3.5	82.2 ± 3.5	17.3 ± 0.0
16%PEG 10,000/ 8%NaPA	28.77	1.57	121	116	4
16%PEG 10,000/ 16%NaPA	29.50	1.45	89	83	6
8%PEG 10,000/ 8%NaPA	29.33	1.37	97.12	91.21	5.91
8%PEG 10,000/ 16%NaPA	30.19	1.28	53.94	46.94	7.00

*The central point of full factorial design was repeated three times.

We also calculated a relative partition of the bacteriocin between the two ATTP systems (P_{Bact}), according to Eq. 6. For all the conditions evaluated except the central point, the P_{Bact} presented values higher than one, as expected base on the higher partition coefficients in the presence of ChCl.

Overall, values of $\%AB_{Bact}$ closer to 100% were obtained in the presence of ChCl, with only two values less than 75% at the following conditions: 8 wt% PEG 2,000/16 wt% NaPA with $\%AB_{Bact}$ = 66%; and 8 wt% PEG 10,000/16 wt% NaPA with $\%AB_{Bact}$ = 54%. In terms of bacteriocin recovery on the top phase, high values (above 75%) were obtained, except for the

conditions at which $\%AB_{Bact}$ was low, probably indicating bacteriocin denaturation at this PEG (top-phase) concentration. Values of $\%REC_{top}$ of approximately 100% were observed with 16 wt% PEG 2,000 or 10,000/8 wt% NaPA systems.

The parameters $\%AB_{Bact}$ and $\%REC_{top}$ were also analyzed as response variables for the PEG/NaPA/cholinium-based salt. The statistical analysis for $\%AB_{Bact}$ and $\%REC_{top}$ are presented in Figure 3.6 and 3.7, respectively, and showed that the independent variables PEG concentration (wt%) and molecular weight of PEG (g/mol) were statistically significant for $\%AB_{Bact}$ and $\%REC_{top}$. An analysis of variance (ANOVA) for these parameters was performed (Table 3.5 and 3.6) and both PEG concentration and molecular weight were significant for the bacteriocin partitioning in ATPPS.

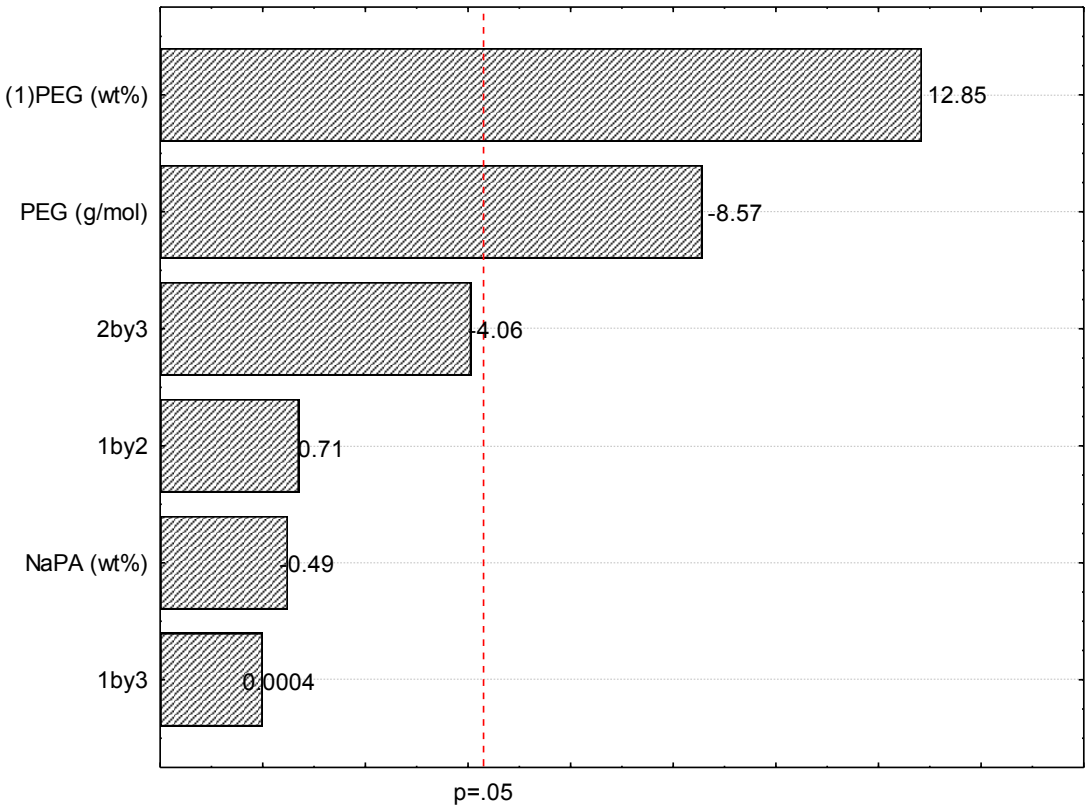


Figure 3.6. Pareto chart of ANOVA applied to identify the variables and interactions with significant effect on $\%AB_{Bact}$ in PEG/NaPA/[Ch]Cl systems. The length of each bar is related to the standardized effect or interaction and the vertical line corresponds to the significant effect for a confidence interval of 95%.

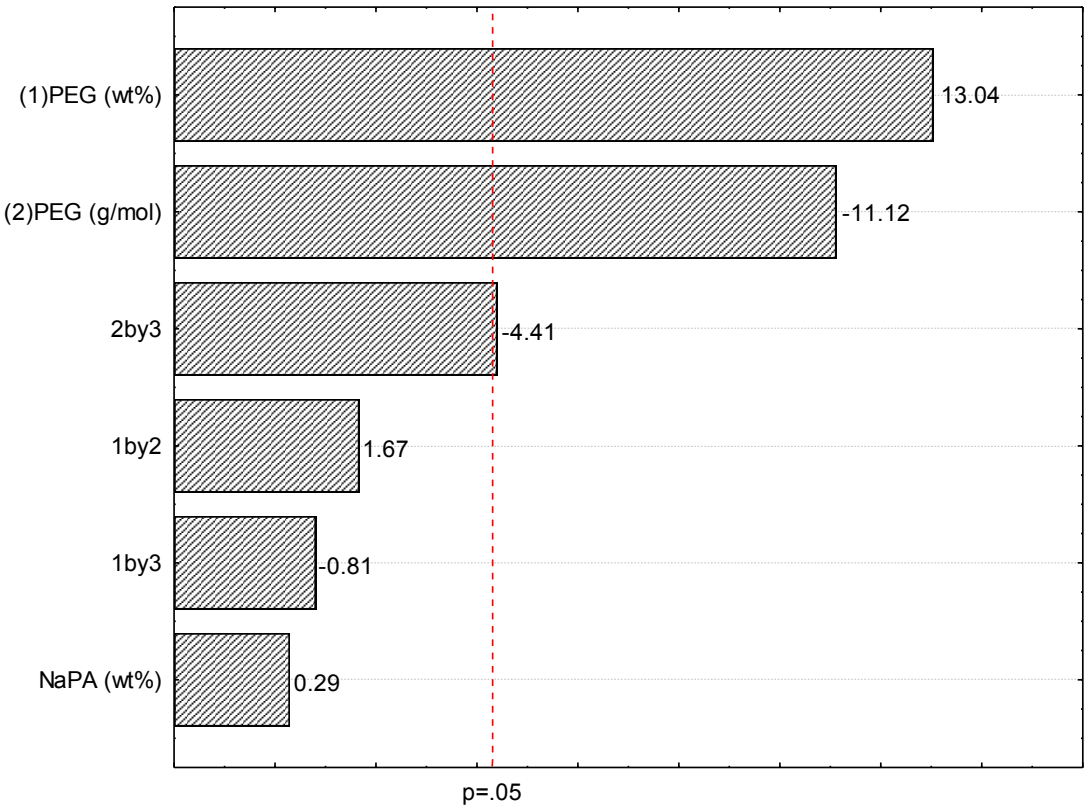


Figure 3.7. Pareto chart of ANOVA applied to identify the variables and interactions with significant effects on $\%REC_{top}$ in PEG/NaPA/[Ch]Cl systems. The length of each bar is related to the standardized effect or interaction and the vertical line corresponds to the significant effect for a confidence interval of 95%.

Table 3.5. Analysis of variance (ANOVA) for the dependent variable $\%AB_{Bact}$ of bacteriocin partitioning in PEG/NaPA/[Ch]Cl systems.

Source	*SS*	*df*	*MS*	*F*	*p*
(1) PEG (wt%)	2032.55	1	2032.55	165.09	*0.006
(2) PEG (g/mol)	904.36	1	904.36	73.45	*0.013
(3) NaPA (wt%)	2.98	1	2.98	0.24	0.671
1 by 2	6.20	1	6.12	0.50	0.552
1 by 3	0.00	1	0.00	0.00	0.999
2 by 3	203.20	1	203.20	16.50	0.056
Pure Error	24.62	2	12.31		
Total SS	3721.77	10			

*Significant level $p < 0.05$; $R^2 = 85\%$. SS = Sum of squares; df = degrees of freedom; MS = Mean squares.

Table 3.6. Analysis of variance (ANOVA) for the dependent variable %REC_{top} of bacteriocin partitioning in PEG/NaPA/[Ch]Cl systems.

Source	*SS*	*df*	*MS*	*F*	*p*
(1) PEG (wt%)	2095.03	1	2095.03	170.17	*0.006
(2) PEG (g/mol)	1523.29	1	1523.29	123.73	*0.008
(3) NaPA (wt%)	1.07	1	1.07	0.09	0.796
1 by 2	34.47	1	34.47	2.80	0.236
1 by 3	8.14	1	8.14	0.66	0.502
2 by 3	239.02	1	239.02	19.41	*0.048
Pure Error	24.62	2	12.31	24.62	
Total SS	3986.79	10			

*Significant level $p < 0.05$; $R^2 = 95\%$. SS = Sum of squares; df = degrees of freedom; MS = Mean squares.

Overall, the best results of bacteriocin partitioning were obtained with higher concentrations of both polymers. For both polymeric aqueous systems, the volume ratio was less than 1, indicating that PEG phase had a smaller volume compared with NaPA phase. For the PEG/NaPA/Na_2SO_4 systems, the response variables (%AB_{Bact} and %REC_{top}) were also analyzed but no statistically significant differences were found.

3.4 Comparison between the PEG/NaPA systems

Analyzing the values of K_{Bact} for both systems, the bacteriocin partition followed the trend: [Ch]Cl > Na_2SO_4. According to Pereira et al. [12], for clavulanic acid partitioning in PEG/NaPA/Na_2SO_4 the entropic driving force cannot play an important role since this molecule partitions to the PEG-rich phase, in spite of this phase being more concentrated in polymer. This observation can be in agreement with our results and to understand the forces that drive the bacteriocin partitioning behavior, the conductivity and pH of each phase after the separation were analyzed. Moreover, we carried out a new series of experiments using the PEG/NaPA/[Ch]Cl system at the best conditions (8 wt% PEG 10,000/8 wt% NaPA/[Ch]Cl), which corresponded to the best combination of partitioning parameters (K_{Bact}, %AB_{Bact}, and %REC_{top}), but now varying electrolyte concentration (0.2 and 0.5 M). A summary of the results is presented in Table 3.7.

Table 3.7. Experimental values of conductivity and pH of top (PEG-rich) phase (TP) and bottom (NaPA-rich) phase (BP) after separation. The partitioning parameters of bacteriocin in PEG/NaPA/salt systems, prepared in sodium acetate buffer at pH 5.0 at 25°C, are also presented.

8%PEG 10,000/ 8%NaPA/salt systems	Phases	Conductivity (mS/cm)	pH	K_{Bact}	$\%AB_{Bact}$	$\%REC_{top}$	$\%REC_{bot}$
0.35 M of Na_2SO_4	TP	0.46	5.45	21.34*	71.66*	60.65*	11.01*
	BP	3.2	5.65				
0.35 M of [Ch]Cl	TP	0.38	5.45	29.33**	97.12**	91.21**	5.91**
	BP	4.1	5.75				
Additional PEG/NaPA/[Ch]Cl systems evaluated							
0.2 M of [Ch]Cl	TP	0.41	5.50	25	95.85	89.64	6.21
	BP	3.6	5.55				
0.5 M of [Ch]Cl	TP	0.3	5.45	32	99.21	93.36	5.85
	BP	4.7	5.80				

* and ** results were already showed in Tables 1 and 2, respectively.

The low values of electrical conductivity and the higher viscosity (data not shown) in the PEG-rich phase (TP) indicate a large difference in total salt and polymer concentration in each phase. The values are in agreement with data by Johansson et al. (2008ab) and Pereira et al. (2012) according to which the PEG-rich phase contains higher total polymer concentration than the bottom phase. The sulfate (SO_4^{2-}) and chloride (Cl^-) anions are a disruptive ions, also called chaotropic ions (in the Hofmeister effect SO_4^{2-} > Cl^- according to Collins and Washabaugh (1985), and can break apart "icebergs" of water molecules that are present in bulk water; this increases the concentration of free water molecules that are able to form hydrogen bonds with nonionic polyethylene oxide (PEO) chains [40]. Taking this into consideration as well as the fact that the partition process in a PEG/NaPA system is thermodynamically favorable (Santos-Ebinuma et al., 2015), it is likely that during the phase separation there is also preferential partitioning of ions to the bottom (NaPA-rich) phase. Since bacteriocin is a small (peptide) molecule (molecular size probably with ~3.5 kDa based on discussion previously mentioned) it is only weakly affected by the entropic exclusion effect. The results indicate that the hydrophobic and electrostatic interactions drive bacteriocin partitioning to the PEG-rich phase. These results can also be confirmed based on the additional PEG/NaPA/[Ch]Cl systems with 0.2 and 0.5 M of [Ch]Cl (Table 6). It is clear that the increase in [Ch]Cl concentration increases the conductivity (0.5 > 0.35 > 0.2 M) in the bottom (NaPA-rich) phase (BP), consequently more bacteriocin partition to the top (PEG-rich) phase.

Bacteriocin extraction was previously evaluated in PEG/salts based ATPPS. To

improve our discussion, we summarize the main results obtained by ATPPS in Table 3.8.

Table 3.8. Partitioning parameters of bacteriocin from different microorganisms by ATPPS obtained in several conditions found in the literature.

ATPP systems employed	Antimicrobial peptide studied and producer microorganism	K_{Bact}	$\%REC_{top}$	Reference
26.5 wt% PEG 8,000/ 11 wt% sodium citrate at pH 7. Centrifugation for 10 min at 2860 x*g*	Bacteriocin from *Pediococcus acidilactici* Kp10	5	83	Abbasiliasi et al., 2014
19 wt% PEG 8,000/ 14 wt% sodium citrate at pH 7 for 30 min		10.73	70.3	Sidek et al., 2016
11 wv% PEG 20,000/ 3.5 wv% $MgSO_4.7H_2O$ at pH 3.0 at 30°C for 40 min	Nisin from *Lactococcus lactis* ATCC 11454	<2	–	Li et al., 2000
15.99 wt% PEG 4,000/15.85 wt% Na_2SO_4 at pH 2. The systems were centrifuged at 30°C.		–	110.17	Li et al., 2001
20 wv% PEG/20 wv% $(NH_4)_2SO_4$ system at pH 7.0. Centrifugation for 10 min at 3,000x*g* after systems were placed at 4°C for 3 h	Cerein 8A from *Bacillus cereus* 8A	9	81	Lappe et al. 2012

"–" results not presented by the authors.

Despite the interesting results found in ATPPS for the purification of bacteriocin from different microorganisms, the main disadvantages were the high PEG and salts concentrations applied for the ATPPS, in some cases more than the double used in our experiments. Also, the results presented by these groups showed lower values for all partitioning parameters in comparison to our data. PEG/NaPA is one of the most promising combinations for ATPPS with electrolytes. PEG/NaPA/salts systems resulted in high recovery yields and proved to be a rapid (only 1 h) and convenient method for bacteriocin extraction from fermented medium.

When both polymers are mixed together, they phase separate at quite low polymer concentrations and at rather mild salt concentrations (Johansson et al., 2011). Another positive aspect is the possibility of recycling the polymers, *i.e*. PEG through salting-out and NaPA through precipitation at pH below 3.

In order to evaluate the purification of bacteriocin in respect to the total proteins in the fermented broth, the partition coefficient of total proteins was also calculated for the best ATPPS, *i.e*. 8 wt% PEG 10,000/8 wt% NaPA with 0.5 M of [Ch]Cl (Table 6). The protein partition coefficient (K_P = 0.51 ± 0.07) shows that the proteins present in the fermented broth are preferentially concentrated in the bottom (NaPA-rich) phase. Thus, our results are very promising since ideally, in a purification process, proteins should partition oppositely to the target molecule, in our case the bacteriocin that partitioned preferentially to the top (PEG-rich) phase.

In addition, the salts used in this study, a neutral salt (Na_2SO_4) and an ionic liquid salt ([Ch]Cl) were very mild for the target molecule. Furthermore, for the [Ch]Cl salt when applied in ATPPS, traces of [Ch]Cl in the bacteriocin extracted by PEG/NaPA/[Ch]Cl ATPPS would not be a concern since choline has been widely used as feed additive for decades (Zeisel & Costa, 2009), as well as for the bacteriocins produced by lactic acid bacteria.

4. Conclusion

The stability and partitioning of bacteriocin from the fermented broth of *Lactobacillus plantarum* ST16Pa in the PEG/NaPA/electrolytes aqueous two-phase systems was studied. Bacteriocin showed high stability after 1 h in temperatures from 50°C to 80°C and pH from 3.0 to 8.0, as well as in the presence of ATPPS components (with exception of 20 wt% NaPA for 24 h that corresponded to a loss of activity of 53%). All the partitioning studies show that the bacteriocin partitions preferentially to the PEG-rich (top) phase (K_{Bact} > 8.9), demonstrating that hydrophobic and electrostatic interactions are the major driving forces of partitioning. The highest partition coefficient (K_{Bact} = 32) was obtained with 8 wt% PEG 10,000/8 wt% NaPA/0.5 M [Ch]Cl, at the same time total proteins were removed in the NaPA-rich (bottom) phase (K_P = 0.51). Our results show the potential of ATPPS with ionic liquids as an initial step for bacteriocin recovery and purification by applying a low-cost (low polymer content) and eco-friendly (mild conditions) PEG/NaPA/ChCl system. Complementary studies on chromatographic processes after bacteriocin recovery in ATPPS are being conducted for high-resolution bacteriocin purification, aiming at its complete characterization.

CHAPTER IV

Enhancement of bacteriocin ST16Pa production by *Lactobacillus plantarum* ST16Pa in a supplemented whey powder formulation proposed by a Plackett & Burman experimental design

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ABSTRACT

Whey is the main byproduct produced by dairy industries. This byproduct is frequently disposed in the environment without any treatment, since this process is extremely expensive. Alternatively, whey can be used as a culture medium to cultivate lactic acid bacteria (LAB) and produce value-added products, such as bacteriocins. Based on this, we aimed to improve the bacteriocin ST16Pa inhibition zones through culturing *L. plantarum* ST16Pa in a whey powder formulation supplemented with additional sources of carbon, nitrogen and vitamin, varying the agitation applied to the system according to a proposed Plackett & Burman (PB) statistical experimental design. This tool shown that tryptone is the only important variable in the production of bacteriocin ST16Pa antimicrobial activity. However, it was not possible to verify if this is depends on another factor. Nevertheless, through the PB design, was possible to obtain a supplemented whey formulation (composed of 150 g/L of whey total solids plus 10 g/L of tryptone and soybean extract in stirred fermentation at 150 rpm) to culture *L. plantarum* ST16Pa and produce a cell-free supernatant (CFS) with antimicrobial activity of 13.23 mm inhibition zone against the biondicator strain. These results are quite significant, since in a previous study, the same strain cultured in cheese whey did no produced antimicrobial activity. In addition, tests performed in our study suggest that the antimicrobial activity produced by the CFS resulted from the supplemented whey powder formulation is the same produced in Man Rogosa and Sharpe (MRS) by other authors, showing equally antimicrobial effectiveness against pathogens.

Keywords: whey powder, supplementation, bacteriocin*, Lactobacillus plantarum,* Plackett & Burman, antimicrobial activity

1. Introduction

Whey is the major byproduct produced by dairy industries, which is obtained after clotting and casein removal during cheese manufacturing (Brandelli, Daroit, & Corrêa, 2015). For many years, industries sought out it cheapest disposal methods, which have usually involved discharge into waterways, ocean, municipal sewage treatment works, and/or onto fields. These practices are completely irresponsible and lead to many environmental and health issues, not only due to the high organic load in whey, but also because of its elevated volume daily produced (Brandelli et al., 2015; Smithers, 2015). When dumped in lands, it creates severe pollution concerns for the surrounding environment by affecting the physicochemical characteristics of soil that result in decreased crop yields. Its discharge into water bodies reduces the dissolved oxygen and hampers biodegradability, which represents a risk to aquatic life (González-Siso, 1996). Nowadays, in most countries, the disposal of untreated whey into waterways or like is unlawful (Smithers, 2015). For that reason, proper management of this byproduct is required before its discharge (Yadav, Yana, Pilli, Kumar, Tyagi, & Surampalli, 2015). Biological wastewater treatment technologies are the safer disposal of whey, but this is an expensive methodology (Panesar, Kennedy, Gandhi, & Bunko, 2007). An economical and advantageous alternative is utilizing it as a substrate for the production of value-added products thought biological means, since this material retain about 55% of total milk nutrients (Panesar et al, 2007; Prazeres, Carvalho, & Rivas, 2012). Among the most abundant nutrients are lactose, soluble proteins, lipids and mineral salts, which are essential for microbial growth, especially for LAB (Koutinas et al., 2009; Panesar et al, 2007).

The LAB are widely used microorganism in food industries acting in the development of meat products, vegetables and various dairy products (Cintas et al., 2001). The LAB, besides playing an important role in food fermentation and demonstrating health beneficiates for consumers, are know for their potential to produce several biomolecules of industrial interest, such as bacteriocins (De Vuyst & Vandamme, 1994).

Bacteriocins are defined as bacterially produced, small, heat-stable peptides that are active against other bacteria and to which the producer has a specific immunity mechanism (Cotter et al., 2005). Although they can be produced by a multitude of microorganisms, bacteriocins produced by LAB has been received increased attention over the past decades due to their safety for human consumption, being considered as *Generally Recognized as Safe* (GRAS) (Balciunas et al., 2013).

Among LAB, the largest group is the genus *Lactobacillus*, which comprises more than 150 different species (Siezen et al., 2010). The specie *Lactobacillus plantarum* is an especially one since it can be adapted to various niches thanks to its ability to ferment a wide range of carbohydrates (Sabo et al., 2014).

Despite whey supports most LAB growth (Brinques et al., 2010; Guerra & Pastrana, 2001; Panesar, Kennedy, Knill, & Kosseva, 2010; Ünlü, Nielsen, & Ionita, 2015), this byproduct may lacks sufficient nitrogen, which often requires its supplementation, not only with nitrogen source but also with other nutritional components, to obtain satisfactory bacteriocin productivities (Briczinski & Roberts, 2002). Moreover, generally, some LAB have an inefficient proteolytic system and are highly dependent on external supplements to synthesize peptides and proteins (Pleissner & Venus, 2016; Ummadi & Curic-Bawden, 2010). In a previously study of Todorov et al. (2011), the researches demonstrated the ability of the *L. plantarum* ST16 to produce high antimicrobial activity against several microorganisms when the strain was cultivated in Man, Rogosa and Sharpe (MRS) broth supplemented with different substrates. However, when it was cultured in cheese whey, in spite of the satisfactory biomass production, this strain did not produce bacteriocin. Therefore, propose culture supplementations to improve the bacteriocin ST16Pa production utilizing an industrial byproduct as whey, could be interesting from the economical point of view. Based on this, we aimed to improve the bacteriocin ST16Pa production through culturing *L. plantarum* ST16Pa in a whey powder formulation supplemented with additional sources of carbon (10, 20 or 30g/L), nitrogen (5 or 10 g/L) and vitamin (0.8 or 1.6 mg/L), varying the agitation (50, 100 or 150 rpm) applied to the system according to a proposed Plackett & Burman (PB) statistical experimental design.

2. Materials and methods

2.1. Microbial cultures

This study was conducted with the probiotic bacteriocin-producing strain *Lactobacillus plantarum* ST16Pa, previously isolated by Todorov et al. (2011) from papaya. As an indicative microorganism of bacteriocin antimicrobial activity, it was used the strains *Listeria innocua* 6a CLIST 2865 (AL230/07), isolated from a sausage sample and provided by Coleção de *Listeria* from Fundação Oswaldo Cruz (FioCruz, Rio de Janeiro, Brazil).

The strains *L. plantarum* ST16Pa and *L. innocua* 6a CLIST 2865 were respectively cultured in MRS and Brain Heart Infusion (BHI) broths (both provided by DIFCO, Detroit, MI, USA), incubated at 30 °C on a orbital shaker that was agitated at 100 rpm for 24 h and then cryopreserved adding 20% (v/v) of glycerol into the culture broths. Finally, the cryopreserved strains were stored at -70°C.

2.2. Enhancement of bacteriocin ST16Pa production

The PB statistical experimental design was used to evaluate the culture conditions and the nutrients that could have higher influence on bacteriocin production by *L. plantarum* ST16Pa cultured in whey powder. For that, 8 variables were screened in 12 trials, with quadruplicate at the central point. The minimal and maximal ranges selected for the 8 factors are presented in Table 4.1. Each column represents an independent variable and each row represents a trial. All tests were carried out in triplicate and the average inhibition zone produced by the bacteriocin ST16Pa was used as the response variable. The biomass produced by *L. plantarum* ST16Pa are also represented in Table 4.1.Variables with confidence levels >95% were considered to have significant influence on bacteriocin ST16Pa inhibition zones. Such experimental design is a two-level partial factorials, most commonly employed for identifying important factors for further investigation (Keneddy & Krouse, 1999). According to these authors, it is a good methods for investigate the effect of many factors and optimize the media compositions and culture conditions in fermentation processes at low cost and through a minimal number of experiments.

Table 4.1. Plackett & Burman experimental design matrix for improvement of inhibition zones produced by bacteriocin ST16Pa in supplemented whey powder, against the bioindicator strain *L. innocua* 6a CLIST 2865. The biomass obtained after 48 h cultivation are also represented.

Runs	[a]Factors/levels								Average inhibition zones (mm)	Biomass (g/L)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8		
1	+1	-1	+1	-1	-1	-1	+1	+1	0.00	0.36
2	+1	+1	-1	+1	-1	-1	-1	+1	0.00	0.31
3	-1	+1	+1	-1	+1	-1	-1	-1	0.00	0.58
4	+1	-1	+1	+1	-1	+1	-1	-1	11.82	0.68
5	+1	+1	-1	+1	+1	-1	+1	-1	0.00	0.32
6	+1	+1	+1	-1	+1	+1	-1	+1	11.56	1.63
7	-1	+1	+1	+1	-1	+1	+1	-1	12.00	0.93
8	-1	-1	+1	+1	+1	-1	+1	+1	0.00	0.43
9	-1	-1	-1	+1	+1	+1	-1	+1	11.37	0.88
10	+1	-1	-1	-1	+1	+1	+1	-1	13,23	1.75
11	-1	+1	-1	-1	-1	+1	+1	+1	11,36	0.51
12	-1	-1	-1	-1	-1	-1	-1	-1	0.00	0.17
13	0	0	0	0	0	0	0	0	11.38	0.50
14	0	0	0	0	0	0	0	0	11.53	0.41
15	0	0	0	0	0	0	0	0	11.58	0.49
16	0	0	0	0	0	0	0	0	11.49	0.46

[a]The factors encoded by +1, 0 and -1, are respectively represented by: X_1 – whey powder total solids: 150, 100, and 50 g/L; X_2 – inulin: 20, 10, and 0 g/L; X_3 – sucrose: 30, 15, and 0 g/L; X_4 – glucose: 30, 15, and 0 g/L; X_5 – soybean extract: 10, 5, and 0 g/L; X_6 – tryptone: 10, 5, and 0 g/L; X_7 – B_{12} vitamin: 1.6, 0.8, and 0 mg/L; X_8 – agitation: 150, 100, and 50 rpm.

The choice to supplement whey powder with glucose (Inlab, São Paulo, Brazil), sucrose (Cromoline Química Fina, São Paulo, Brazil), triptone (DFICO, Detroit, USA) and vitamin B_{12} (Inlab, São Paulo, Brazil) was based on the study of Todorov et al. (2011). Additionally, we also tested inulin (Clariant, São Paulo, Brazil), as a well-known compound to increase cell growth of probiotic bacteria. Besides, in order to promote the improvement of inhibition zones produced by bacteriocin ST16Pa at an attractive and economical level, we used soybean extract (Jasmine, Curitiba, Brazil), an agro-industrial compounds with high protein concentration. Considering that Brazil is the second largest producer of this grain (Folha de São Paulo, 2017), whey supplementation using this substance as nitrogen source is additionally advantageous. A whey powder formulation without supplementation was

prepared with the conditions setted up in the central point of the PB experimental matrix (100 g/L of total whey solids; 30 °C; 100 rpm – see Table 4.1), was used as control.

2.3. Whey preparation

Whey powder, kindly provided by BR Foods (São Paulo, SP, Brazil), was used to carry out the investigations in this work. In order to optimize the inhibition zones produced by bacteriocin ST16Pa in this byproduct, a PB experimental design was proposed. According to the PB experimental matrix (Table 4.1), whey was prepared using different concentration of total solids, which were diluted in distillated water. According to the methodology of Guerra and Pastrana (2001) and Vignolo, Kairuz, Holgado, and Oliver (1995), the pH of whey-base solution was acidified to 4.0 by addition of 10 M HCl and heated at 100 °C for 30 min in thermal bath in order to denature proteins and remove insoluble contents. The resulted solution was centrifuged and the whey-base supernatant was adjusted at pH 6.3 using 10 M NaOH. This supernatant was subsequently supplemented with the additional carbon, nitrogen and vitamin sources in accordance with the proposed PB experimental matrix (Table 4.1). Finally, it was pasteurized at 90 °C for 5 min in a thermal bath and subsequently cooled in ice bath for 10 min.

2.4. Inoculum preparation and culture conditions

L. plantarum ST16Pa inoculum was prepared adding 1 mL of culture stock into Erlenmeyer flask (250 mL) containing 100 mL of MRS broth (DIFCO, Detroit, MI, USA) and incubated in orbital shaker at 30 °C at 100 rpm for 12 h. Then, the inoculum size was adjusted with sterile distilled water to a final concentration of 0.8 – 0.9 optical density (O.D) per mL at 650 nm. The cells were harvested by centrifugation at 4,470 *g* for 15 min at 4 ° C. The cell *pellet* was washed twice with sterile distilled water and resuspended directly in previously treated and supplemented whey.

Ten mL of inoculum (10% [v/v] of the final volume to be fermented) was transferred into Erlenmeyer flasks (250 mL) containing 90 mL of previously treated and supplemented whey powder. All cultures were performed in triplicate and incubated at 30 °C for 48 h in an orbital shaker at 50, 100 or 150 rpm, in accordance with the proposed PB experimental matrix (Table 4.1). In triplicate, samples were collected after this period to analyze cell growth and the production of inhibition zones by bacteriocin ST16

2.5. Scaling up in bioreactor and fermentative parameters

On the basis of the data obtained thought the assays proposed by the PB experimental design, the scale up of the supplemented whey powder formulation, which generated the best results regarding inhibition zones by bacteriocin ST16Pa and biomass, was carried out.

A typical stirred tank bioreactor (Biostat B plus, Sartorius) with capacity for 2 L was used to conduct this experiment. To this end, 0.9 L of supplemented powder whey, prepared according to previously established conditions (see Table 4.1), was transferred to the tank and subsequently added with 100 mL of inoculum (10% [v/v] of the final working volume). In triplicate, the cultures were conducted as same as conditions setted up in the assay developed in orbital shaker.

Samples were collected every 2 h during 12 h, and then, after 24 and 48 h of fermentation. Each sample was analyzed regarding *L. plantarum* ST16Pa cell growth, inhibition zones produced by bacteriocin ST16Pa and lactate production, as well as consumed lactose. Thought the respective obtained values, fermentative parameters, such as lactose to biomass yield, lactose to lactate yield, global volumetric productivity of biomass, global volumetric productivity of lactate, maximum specific growth rate and generation time, were calculated according to the Equations 1 to 6, respectively, as follow:

$$Y_{\frac{biomass}{lactose}} = \frac{(X_f - X_i)}{(S_f - S_I)} \quad \text{(Equation 1)}$$

$$Y_{\frac{lactate}{lactose}} = \frac{(P_{LACf} - P_{LACi})}{(S_f - S_i)} \quad \text{(Equation 2)}$$

$$Q_{biomass} = \frac{(X_f - X_i)}{T_f} \quad \text{(Equation 3)}$$

$$Q_{lactate} = \frac{(P_{LACf} - P_{LACi})}{T_f} \quad \text{(Equation 4)}$$

$$\mu_{max} = \frac{1}{(T_f - T_i)} ln \frac{X_f}{X_i} \quad \text{(Equation 5)}$$

$$T_g = \frac{ln2}{\mu_{max}} \quad \text{(Equation 6)}$$

In order to evaluate the reproducibility in bioreactor, these parameters were compared to those obtained in the assay carried out in orbital shaker using the same condition and the same supplemented whey powder formulation.

2.6. Determination of lactose and lactate concentration

To determine the concentration of lactose and lactate, samples were aseptically collected and centrifuged at 3,421 *g* for 15 min. The supernatant was filtered through 0.22 μm pore membranes (Millipore, Bedford, MA, USA), transferred to collection vials and determined by High Performance Liquid Chromatography (HPLC) divise (Ultimate 3000, Dionex, Sunnyvale, CA, USA), equipped with a refractive index detector (Shodex RI-210, Kawasaki, Kanagawa, Japan) and a HPX-87H column (Bio-Rad, Hercules, CA, USA). Analyses were carried out at 50 °C, using 5.0 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min. High purity lactose and lactate (Sigma-Aldrich, St. Louis, MO, USA) were used at concentrations from 0.1 to 10.0 g/L as standard solutions to prepare the calibration curve.

2.7. Cell growth

The *L. plantarum* ST16Pa growth was monitored by counting colony-forming units (CFU) per mL using the serial dilution technique, in which 0.5 mL of the sample was serially 10-folds diluted in 4.5 mL of 0.85% (w/v) sterile saline. Subsequently, 100 μL of each dilution was transferred to Petri dishes (90 x 15 mm) containing MRS broth solidified with 1.5% (w/v) of agar. With the aid of a sterile Drigalski handle, the diluted samples were spread over the surface of the medium plates. The plates were incubated for 48 h and only those containing 30 to 300 colonies were considered.

To convert the CFU values to cells dry mass (g/L), *L. plantarum* ST16Pa strain was cultivated in MRS broth under recommended conditions (30 °C/100 rpm), according to Todorov et al. (2011). By associating the CFU values and the corresponding cells dry mass obtained by filtration of the MRS broth in a 0.22 μm pore membrane (Millipore, Bedford, MA, USA), a calibration curve was generated, where the supplied equation ($y = 8.10^7x\text{-}1.10^7$, $R^2 = 0.98$) served as basis to convert the CFU values of the cultures in the treated whey powder for cells dry mass values in g/L. The CFU/mL determination was performed in triplicate.

2.8. Bacteriocin antimicrobial activity assay

For bacteriocin ST16Pa antimicrobial activity determination, in triplicate, samples were centrifuged at 25,750 *g* at 4 °C for 10 min and the cell-free supernatants (CFS) were

performed according to spot-on-the-lawn method. For this, petri dishes containing 10 mL of melted BHI soft agar (supplemented with 0.75% of agar [w/v]) was previously inoculated with 1 mL of overnight bioindicator microorganism diluted 100-folds (approximately 10^8 CFU/mL). After solidify, 20 µL of CFS was dropped onto the BHI soft agar surface and incubated at 30 °C for 24 h. Next, using a digital caliper (Lee Tools, model 684132), the inhibition zones were measured in 4 different directions and the avarege values (in mm) were considered. Further, the antimicrobial activity produced by whey powder formulation, which obtained the higher bacteriocin ST16Pa inhibition zones, was quantified by serial 2-fold dillution of CFS in 25 mM phosphate buffer at pH 6.5. The amount of bacteriocin produced by *L. plantarum* ST16Pa in the supplemented whey powder formulation was expressed in arbitrary units per mL (AU/mL) by the equation $AU/mL = D^n \times 1000/P$, where D = dilution factor, n = first dilution not showing any inhibition zone, and P = volume (µL) of supernatant deposited onto the agar surface.

2.9. Bacteriocin ST16Pa inhibition zones stability

In order to prove that the antimicrobial activity produced in the supplemented whey powder has the same protein nature and stability as described by Todorov et al. (2011) culturing *L. plantarum* ST16Pa in MRS broth, the effect of digestive enzymes, chemicals and temperature on bacteriocin ST16Pa inhibition zones were investigated. For this, the CFS obtained from the scaled up supplemented whey powder formulation was treated adjusting the pH to 6.0 with 1 N NaOH. Aliquots of 1 mL were incubated at 30 °C for 2 h added with 1.0, 5.0 or 30 mg/mL of chymiotrypsin, trypsin, protease XIV and α-amylase (all purchased from Sigma-Aldrich, MO, USA). In a separate test, another 1 mL of CFS was individually treated with 1% (w/v) of sodium dodecyl sulfate (SDS), urea, EDTA and NaCl or with 1% (v/v) of Triton X-100 Tween 20 and Tween 80. Next, the solutions were incubated at 30 °C for 1 h. The effect of temperature on bacteriocin ST16Pa inhibition zones was tested by heating the CFS in a thermoregulated bath at 40, 60, 80, and 100 °C for 1 h and at 121°C for 20 min. All the aforementioned studies were tested for antimicrobial activity against the bioindicator strain *L. innocua* 6a CLIST 2865 using the spot-on-the-lawn method as described before. Untreated CFS with enzyme, chemical and temperature served as controls.

2.10. Effect of bacteriocin ST16Pa on bioindicator microorganism growth

A 4.5 mL aliquot of CFS obtained from the scaled up supplemented whey powder formulation was added to Erlenmeyer flasks (100 mL) containing 30.5 mL of BHI broth previously inoculated with 0.35 mL of overnight-old *L. innocua* 6a CLIST 2865. The control assay was carried out as the same conditions described above, except by adding the CFS. Then, the Erlenmeyer flasks were incubated at 37 °C in orbital shake at 100 rpm for 6 h. Optical density readings (at 600 nm) were recorded every 1 h.

2.11. Reduction of bioindicator viable cells in the presence of bacteriocin ST16Pa

Overnight-old culture of *L. innocua* 6a CLIST 2865 was harvested at 4,470 *g* at 4°C for 15 min., the *pellet* was washed twice with 0,85% (w/v) of sterile saline solution and resuspended in 10 mL of this same solution. Equal volume of the CFS obtained from the scaled up supplemented whey powder formulation was mixed with the resuspended *pellet* and viable cell numbers were determined before and after incubation for 1 h at 37 °C by plating this solution onto BHI broth added with 1% (w/v) of agar. Cell suspensions of the bioindicator strain without adding the CFS served as controls.

2.12. Purification of bacteriocin ST16Pa

A 100 mL aliquot of CFS obtained from the scaled up supplemented whey powder formulation was treated adjusting the pH to 6.0 with 1 M NaOH and subsequently used for further assays. Bacteriocin ST16Pa was precipitated by adding ammonium sulfate to the CFS to obtain 20% (w/v) saturation and, then, stirred for 2 h at 4 °C. After centrifugation for 30 min at 4,470 *g* and 4° C, the resulting *pellet* was resuspended in 10 mL of 25 mM ammonium acetate buffer (pH 6.5) and loaded on a previously activated C_{18} solid phase extraction (SPE) cartridge, named as OASIS® HLB (Waters, Millipore, MA, USA), which was washed with gradual concentration of isopropanol (20, 40, 60 and 80% [v/v]) in the same buffer above mentioned. In order to control and validate the proposed protocol, *L. plantarum* ST16Pa was cultured in MRS broth at 30 °C for 24 h, as recommended by Todorov et al. (2011), and the resulted CFS was treated as previously described.

The antimicrobial activities were tested against *L. innocua* 6a CLIST 2865 using the spot-on-the-lawn method as previously described (see section 2.7 of this chapter). The protein

concentration of each step was determined using the BCA protein assay reagent (Sigma-Aldrich, MO, USA) as specified by the manufacturer and through a calibration curve (y = 0.0011x + 0.00245, R^2 = 0.99), it was possible to calculate the specific activity (AU/mg), yield (%) and purification factor (fold).

2.13. Statistical analysis

The PB experimental designs matrix and the resulted data were analyzed through the Minitab 17 Statistical Software (Inc., State College, PA, USA). Statistical verification of the results was performed by analysis of variance (ANOVA).

3. Results and discussion

3.1. Enhacement of bacteriocin ST16Pa production

Using a PB statistical experimental design, the individual effects of concentrations of whey powder total solids, inulin, glucose, sucrose, soybean extract, tryptone, vitamin B_{12} and the agitation on bacteriocin ST16Pa inhibition zones was evaluated. Table 4.1 shows the obtained inhibition zones against the bioindicator strain and the respective biomass production after culture *L. plantarum* ST16Pa for 48 h in orbital shaker at 30 °C. Before developing the runs proposed by the PB experimental matrix, *L. plantarum* ST16Pa was grown in no supplemented whey powder treated as previously describe in section 2.2 of this chapter. This assay served as control of the strain performance and was equally conducted at 30 °C incubation, jointly with conditions setted up in the central point of this same experimental design (100 g/L of total whey solids at 100 rpm). In this control assay, even the strain showing good adaptation to the whey powder formulation and reasonable growth rates (0.22 g/L), no inhibition zone was produced. This result is equivalent to that reported by Todorov et al. (2011), in which no bacteriocin ST16Pa was detected using cheese whey (20 or 100 g/L of total solids) as fermentative substrate.

Following the PB experimental matrix, from a total of 16 assays, 10 whey powder formulations showed considerable growth rate of *L. plantarum* ST16Pa and satisfactory bacteriocin ST16Pa inhibition zones (runs 4, 6, 7, 9, 10, 11 and 13-16). Biomass production varied markedly in a range between 0.17 and 1.75 g/L and bacteriocin ST16Pa inhibition zones between 0.00 to 13.23 mm, reflecting the importance of optimization to obtain higher

productions. The Pareto chart (Figure 4.1), obtained through the data analysis by Minitab 17 Statistical Software, showed that the tryptone is the only important variable on the bacteriocin ST16Pa inhibition zones, demonstrating significant positive effect at a 95% confidential level. Independently of the tryptone concentration used to supplement the whey powder formulations, all assays in which this compound was part of resulted in bacteriocin ST16Pa inhibitions zones up to 11.00 mm.

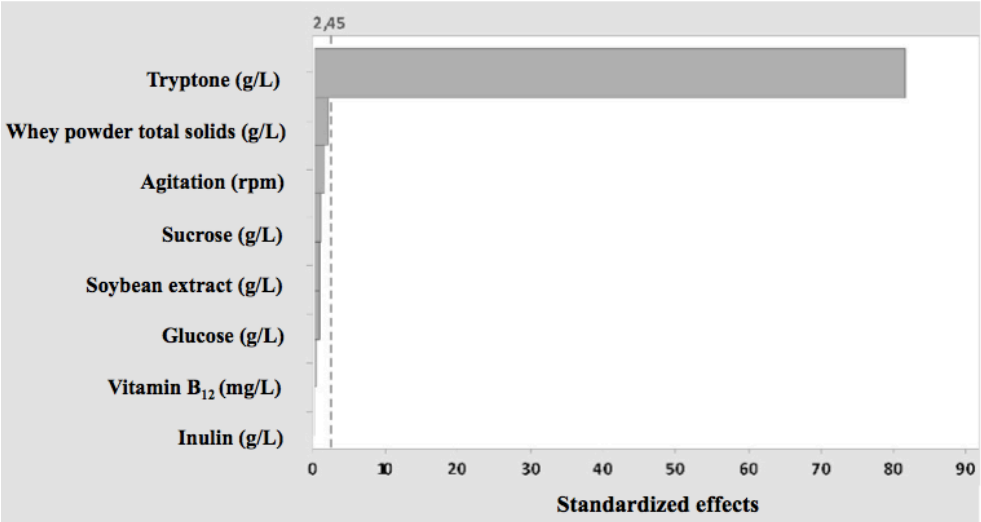

Figure 4.1. Pareto chart demonstrating the individual effects of the variables triptone, whey powder total solids, agitation, sucrose, soybean extract, glucose, vitamin B_{12} and inulin on bacteriocin ST16Pa inhibition zones according to Plackett & Burmann experimental design.

Todorov et al. (2011) also reported increasing in the bactericin ST16Pa production from 25,600 to 51,200 AU/mL when the strain *L. plantarum* ST16Pa was cultured in MRS broth supplemented with tryptone (20 g/L). Our results are also in agreement with those reported for sakacin P production by *Lactobacillus sakei* CCUG 42687 (Aasen, Møretrø, Katla, Axelsson, & Storrø, 2000), bacteriocin ST341LD produced by *L. plantarum* ST341LD (Todorov & Dicks, 2006a), and recently for the bacteriocins produced by *Lactococcus lactis* 19.3 and *Enterococcus durans* 41.2 (Zamfir, Stefan, & Grosu-Tudor, 2016). This occurrence may be associate to the fact that tryptone served as source of various amino acids essential for the biosynthesis of the bacteriocin or acted as inducers of its production (Cabo, Murado, Gonzalez, Vazquez, & Pastoriza, 2001).

In the other hand, when was prepared a whey powder formulation using tryptone as sole ingredient supplementation (3, 10 or 17 g/L, individually), no bacteriocin ST16Pa inhibition zones were produced. This can be explained because the PB statistical experimental design has some drawbacks, which consist of not verify if the effect of one factor depends on

another factor or assumes that the important main effects will be much larger than two-factor interactions (Montgomery, Borror, & Stanley, 1997). Moreover, according to Myer and Montgomery (2002), the main effects can be confused with second order interactions and second order interactions can be confused with each other. Based on this, we suppose that the improved inhibition zones achieved in this work is associated not only to tryptone effect but also to another independent variable, which is not possible to be detected using this type of statistical experimental design. As consequence, the following assays were developed taking into account the whey powder formulation that achieved higher production for both bacteriocin ST16Pa inhibition zones (13.23 mm) and biomass concentration (1.75 g/L) – represented by run #10 –, although the bacteriocin ST16Pa production seems not be essentially associated with the strain growth, as can be observed in Figure 4.2.

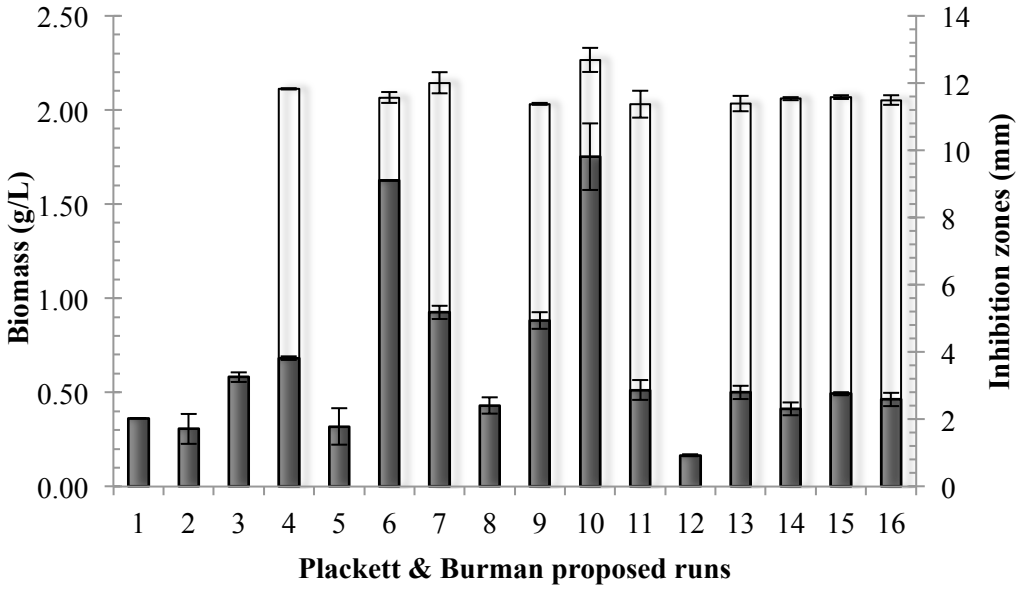

Figure 4.2. *Lactobacillus plantarum* ST16Pa growth (gray bars) and bacteriocin ST16Pa inhibition zones (white bars) resulted from runs proposed by Plackett & Burmann experimental desing.

According to Kim, Hall, and Dunn (1997) and Bogovic-Matijasic and Rogelj (1998), the bacteriocin production is strongly dependent on pH, nutrient sources, incubation temperature, but activity levels do not always correlated with cell mass or growth rate of the producer strain. Additionally, occasionally, the increased levels of bacteriocin production are obtained under sub-optimal conditions (De Vuyst, Callewaert, & Crabbé, 1996).

3.2. Bioreactor scale up and kinetic parameters

Once the cultivation of *L. plantarum* ST16Pa in orbital shaker using the whey powder formulation corresponding to run #10 from PB experimental design resulted in higher bacteriocin ST16Pa inhibition zone and biomass concentration (see Table 4.1), the same condition was scaled up. The values of biomass, bacteriocin ST16Pa activity in AU/mL, lactate concentration, as well as the fermentative parameters obtained in both systems (orbital shaker and bioreactor) are presented in Table 4.2. As can be observed, there are no considerable differences between the parameter values obtained in orbital shaker and bioreactor, being this assay easily scalable.

Table 4.2. Fermentative parameters, biomass production, lactate concentration and bacteriocin ST16Pa activity produced by *L. plantarum* ST16Pa after 48 h cultivation in the selected whey powder formulation (run #10 from Plackett & Burman experimental design) conducted in orbital shaker and bioreactor.

	Orbital shaker	**Bioreactor**
Biomass (g/L)	1.50	1.56
Bacteriocin ST16Pa activity (AU/mL)	1,600	1,600
Lactate (g/L)	8.85	8.61
μ_{max} (h^{-1})	0.48	0.48
T_g (h)	1.44	1.44
$Q_{lactate}$ (g/L.h)	0.18	0.18
$Q_{biomass}$ (g/L.h)	0.03	0.03
$Y_{biomass/lactose}$ (g/g)	0.08	0.07
$Y_{lactate/lactose}$ (g/g)	0.46	0.39

μ_{max}: maximum specific growth rate; T_g: generation time; $Y_{biomass/lactose}$: Lactose to biomass yield; $Y_{lactate/lactose}$: Lactose to lactate yield; $Q_{biomass}$: global volumetric productivity of biomass; and $Q_{lactate}$: global volumetric productivity of lactate (48 h cultivation).

3.3. Effect of proteolytic enzymes, chemicals and temperature on bacteriocin ST16Pa inhibition zones

The CFS used to evaluate the effect of proteolitic enzymes, chemicals and temperature on bacteriocin ST16Pa inhibition zones was that obtained through culturing *L. plantarum* ST16Pa in the scaled up supplemented whey powder formulation – run #10 from PB experimental design. All results from these tests were compared with the initial inhibition zones (13.23 mm) of the CFS against the bioindicator strain and are summarized at Table 4.3.

Table 4.3. Effect of proteolitic enzymes, temperature and chemical compounds on bacteriocin ST16Pa inhibition zones against *L. innocua* 6a CLIST 2865.

Stability	Concentration	Inhibition zones (mm)[a]
Enzymes		
Chymiotrypsin	1 mg/mL (w/v)	-
Trypsin		-
Protease XIV		-
α-amylase		+++
Chymiotrypsin	5 and 30 mg/mL (w/v)	-
Trypsin		-
Protease XIV		-
α-amylase		-
Temperature		
40°C/1 h		+++
60°C/1 h		+++
80°C/1 h		+++
100°C/1 h		++
121°C/20 min.		+
Chemicals		
Triton X-100	1% (v/v)	+
Tween 20		+++
Tween 80		+++
SDS		++++
NaCl	1% (w/v)	+++
Urea		+++
EDTA		+++

[a]Inhibition zones are expressed in: (++++) > 14.0 mm; (+++) 12.5 – 13.9 mm; (++) 11.0 – 12.4 mm; (+) 10.0 – 10.9 mm; and (-) no inhibition zone.

The CFS treated with 1.0, 5.0 and 30 mg/mL of chymiotrypsin, trypsin and protease XIV, resulted in complete inactivation of antimicrobial activity against the bioindicator strain. When treated with 1.0 mg/mL of α-amylase, the CFS inhibition zones decreased but not completely, reaching 13.00 mm. In the other hand, when treated with higher concentrations of the same enzyme (5.0 and 30.0 mg/L), no inhibition zones were observed. Figure 4.3 demonstrates that, when the bacteriocin ST16Pa of CFS is affected by 30.0 mg/mL of all enzymes, the inhibition zones, usually circular, become deformed.

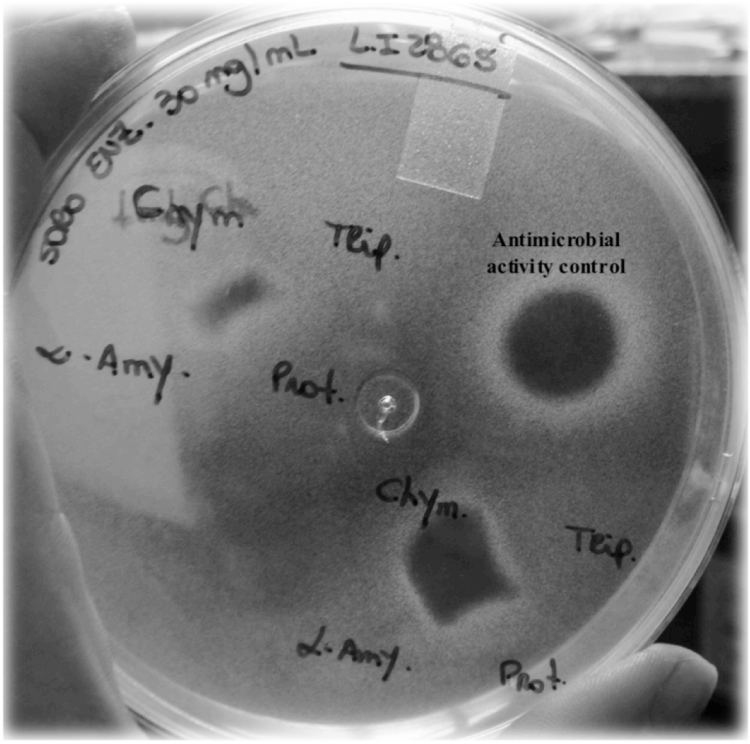

Figure 4.3. Effect of 30 mg/L of the proteolic enzymes chymiotrypsin (chym.), trypsin (trip.), protease XIV (prot.) and α-amylase (α-amy.) on inhibition zones produced by cell-free supernatant obtained from *L. plantarum* ST16Pa cultured in supplemented whey powder formulation (run #10 from Plackett & Burmann experimental design).

Despite antimicrobial inactivation by high concentration of α-amylase, our results corroborated with Todorov et al. (2011), which reported no effect of 1.0 mg/mL of this enzyme on bacterion ST16 stability. The α-amylase is produced by the salivary glands and its main function is to initiate the digestion of macromolecules such as carbohydrates (Cardoso et al., 2017). Based on this information, we also agree with Todorov et al. (2011), who suggested that the bacteriocin ST16Pa does not belong to the controversial group IV of the bacteriocins, which contain carbohydrates or lipids in the active molecule structure.

Regarding the chemical effects, Tween 20, Tween 80, NaCl, urea and EDTA did not affect the inhibition zones produced by the CFS. The same stability is observed for the bacteriocin ST16Pa produced in MRS broth (Todorov et al., 2011), excepted by the effect of SDS and Triton X-100 on our produced bacteriocin ST16Pa showing positive effect with the increase inhibition zone from 13.23 to 15.85 mm, and negative effect, decreasing the inhibition zone from 13.23 to 10.21 mm, respectively. The negative effect of Triton X-100 on bacteriocin stabiliy is also reported by Todorov and Dicks (2005c). A possible hyposthesis for this effect may be related to the interaction effect between the surfactant Triton X-100 and the bacteriocin. Although Triton X-100 is an uncharged surfactant, it can interact with the

bacteriocin changing its tertiary structure to a less active form. For further details, a study of bacteriocin stability in the presence of polymers and salts can be seen in Chapter III.. Regarding the SDS, the positive effect on bacteriocin ST16Pa inhibition zones can be explained because this compound, when 1% (w/v) was diluted in sterile water and served as control, exhibited antimicrobial activity against the strain *L. innocua* 6a CLIST 2865 (inhibition zone of 5.0 mm). Thus, when the CFS was treated by this detergent, a synergistic antimicrobial activity is verified.

The inhibition zones produced by bacteriocin ST16Pa also remained stable after 1 h at thermo bath at 40, 60, and 80 °C. However, a slight decrease in inhibition zones was observed upon heat treatment at 100 °C for 1 h (from 13.23 to 11.28 mm) and at 121 °C for 20 min. (from 13.23 to 10.68 mm). This finding is consistent with results reported for a number of bacteriocins produced by strains of *L. plantarum* (Lee & Paik, 2001; Wen, Philip, Ajam, 2016). This heat stability could be a very useful characteristic as food preservative, because many food-processing procedures involve a heating step (Xie et al., 2011).

3.4. Antimicrobial mode of action of bacteriocin ST16Pa against the bioindicator strain

The CFS, resulted from the scaled up cultivation of *L. plantarum* ST16Pa in supplemented whey powder respective to run #10 from PB experimental design, substantially inhibited the *L. innocua* 6a CLIST 2865 growth, presenting $O.D_{600nm}$ from 0.0 to 0.39 in 6 h of cultivation. The control, in which the biondicator strain was untreated with the CFS, increased the $O.D._{600nm}$ from 0.0 to 1.72 in the same period (Figure 4.4). When the overnight-old cells of the bioindicator strains was treated with the same CFS for 1 h, the viable cell decreased from 9.13 to 8.40 log CFU/mL, suggesting the same bactericidal mode of action reported by Todorov at al. (2011). It is necessary to highlight that this high concentration of *L. innocua* contamination is very unlikely to occur in foods, but the almost 1 log reduction in the contamination level is very important from a public health standpoint.

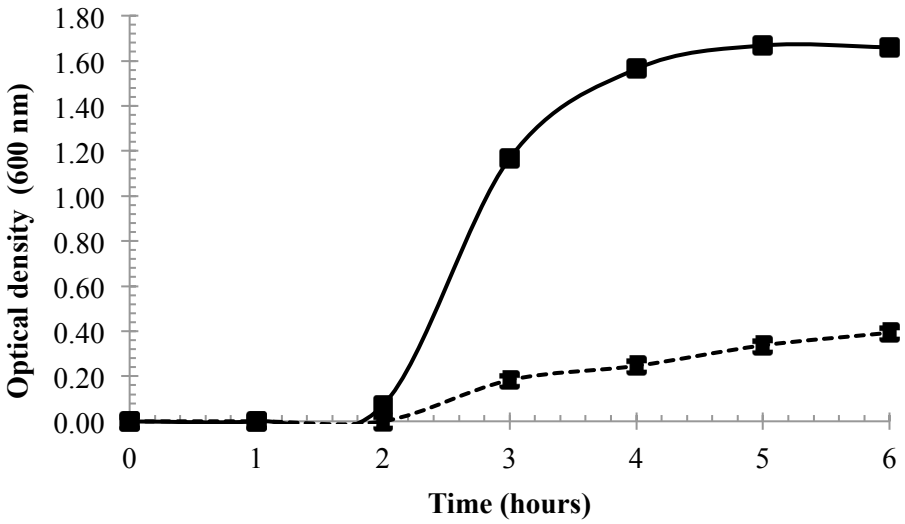

Figure 4.4. Effect of bacteriocin ST16Pa on growth of the bioindicator strain *L. innocua* 6a CLIST 2865 (dotted line). A sample no added with bacteriocin ST6Pa, served as control (continuous line).

3.5. Purification of bactericion ST16 obtained in MRS broth and in supplemented cheese whey formulation

A purification protocol was adopted in order to evaluate a possible increase on bacteriocin ST16 antimicrobial activity of the CFS resulted from the scaled up supplemented whey powder formulation (run #10 from PB experimental design). A CFS obtained from culturing *L. plantarum* ST16Pa in MRS broth served as control to validate the proposed protocol. Both CFS was firstly prepurified by ammonium sulfate precipitation (20% [w/v] saturation), followed by application into previously actived SPE OASIS® HLB cartridge. The bacteriocin ST16Pa produced in MRS broth and in the supplemented whey powder formulation equally eluted with 40% isopropanol in 25 mM ammonium acetate buffer (pH 6.5).

The quantification of bacteriocin ST16Pa antimicrobial activity of each mentioned step, as well as the total activity (AU), total proteins (mg), specific activity (AU/mg), yield (%) and purification fold can be verified in Table 4.4.

Table 4.4. Purification of bacteriocin ST16 obtained from culture in MRS broth and in supplemented whey powder formulation.

Steps	Volume (mL)	Activity (AU/mL)	Total protein (mg)	Total activity (AU)	Specific activity (AU/mg)	Yield (%)	Purification fold
MRS broth							
Cell-free supernatant	100	3.2×10^3	9.09	3.2×10^5	3.5×10^7	100	1
Ammonium sulfate precipitation	10	6.4×10^3	0.91	6.4×10^4	7.0×10^7	20	1.99
SPE OASIS® HLB	3	3.2×10^3	0.02	9.3×10^3	4.8×10^8	3	13.63
Supplemented whey powder formulation							
Cell-free supernatant	100	1.6×10^3	20.93	1.6×10^5	7.6×10^3	100	1
Ammonium sulfate precipitation	10	6.4×10^3	11.37	6.4×10^4	5.6×10^3	40	0.73
SPE OASIS® HLB	2	1.6×10^3	6.19	3.2×10^3	0.5×10^3	2	0.07

The proposed purification protocol worked well for bacteriocin ST16Pa produced in MRS broth. At final step using the SPE OASIS® HLB, despite low yield (only 3%), the purification fold increased approximately 13.7 as compared with the CFS. In the other hand, the same protocol was not effective for purification of the bacteriocin produced in the supplemented whey powder formulation, evidently by the complexity of this medium (2.3-fold more contaminated with total proteins regarding to CFS from MRS broth), thus requiring other previous purification steps or another purification protocol.

4. Conclusions

Although the PB experimental design adopted to develop this work could not the best statistical tool to optimize the bacteriocin ST16Pa production, since it was not able to point out association between tryptone and other variables, through it was possible to obtain a supplemented whey powder formulation, which resulted in greater bacteriocin ST16Pa inhibition zone compared with another research demonstrating no antimicrobial activity by the same strain cultured in cheese whey. In addition, the tests carried out in the present work suggest that the antimicrobial activity produced by the CFS resulted from the cultivation of *L. plantarum* ST16Pa in supplemented whey powder formulation is the same produced in MRS broth by Todorov et al. (2011), showing equally antimicrobial effectiveness against pathogens. Regarding the bacteriocin ST16Pa purification, the proposed protocol is not the most suitable when complex culture medium is used to cultivate the *L. plantarum* ST16Pa. In this case, a liquid-liquid extraction by aqueous two-phase systems can be a prepurification alternative for contaminats removal (total protein), specifically through aqueous two-phase polymer systems with ionic liquids, as proposed in Chapter III.

CHAPTER V[1]

Inhibitory substances production by *L. plantarum* ST16Pa cultured in hydrolyzed cheese whey supplemented with soybean flour and their antimicrobial efficiency as biopreservatives on fresh chicken meat

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ABSTRACT

Cheese whey, the main byproduct of the dairy industry, is one of the most worrisome types of industrial waste, not only because of its abundant annual global production but also because it is a notable source of environmental pollution. However, cheese whey can serve as a raw material for the production of biocomposites. In this context, in this study, we assayed the production of a bacteriocin-like inhibitory substance (BLIS) and lactate by culturing *Lactobacillus plantarum* ST16Pa in hydrolyzed fresh cheese whey. The process was improved by studying the enzymatic hydrolysis of cheese whey as well as its supplementation with soybean flour under microaerophilic or anaerobic conditions. Thus, the highest values of BLIS (7,367.23 arbitrary units [AU]/mL) and lactate yield ($Y_{lactate/lactose}$ = 1.39 g/g) were achieved after addition of 10 g/L soybean flour in microaerophilia. These conditions were successfully scaled up in a bioreactor because during complete anaerobiosis at 150 rpm, *L. plantarum* ST16Pa attained considerable cell growth (3.14 g/L), lactate concentration (14.33 g/L), and BLIS activity (8,082.56 AU/mL). In addition, the cell-free supernatant resulting from this bioprocess showed high biopreservative efficiency in chicken breast fillets artificially contaminated with *Enterococcus faecium* 711 during 7 days of refrigerated storage, thus indicating the potential use of this BLIS as a biopreservative in the food industry.

Keywords: cheese whey, soybean flour, BLIS, biopreservative*, Lactobacillus plantarum*

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1. Introduction

Diseases related to the consumption of food contaminated by pathogens or to food spoilage are some of the most worrisome public health problems in the world (García, Rodríguez, Rodríguez, & Martínez, 2010). In particular, the risk of consumption of meat contaminated with an *Enterococcus* species is associated with this species' intrinsic resistance to various classes of antimicrobial drugs (Bortolaia & Guardabassi, 2015; Bortolaia, Espinosa-Gongora, & Guardabassi, 2016). Once ingested, this microorganism can colonize the human digestive tract and transmit, through horizontal transfer, resistance genes to other microorganisms of the intestinal microbiota, in addition to the risk of transference to important pathogens such as *Staphylococcus aureus* and *Listeria* spp. (Pesavento, Calonico, Ducci, Magnanini, & Lo Nostro, 2014). Therefore, the control of contamination of poultry meat by this bacterium is necessary.

The globalization of the food industry, new manufacturing processes, and the increasing demand for minimally processed foods can directly affect the incidence of these diseases (Anacarso et al., 2014). For this reason, there is substantial concern about the preservation and safety of food (Bali, Panesar, & Bera, 2014). Among the existing technologies, biopreservation, through the controlled use of certain microorganisms or their metabolites in order to extend shelf life, is given increasing attention owing to its minimal impact on the nutritional and sensory properties of perishable food products (García et al., 2010). Biopreservation is generally carried out through fermentative processes using lactic acid bacteria (LAB), which have a high potential as biopreservatives because they are safe for human consumption and because they produce various antimicrobial compounds such as organic acids, bacteriocins, or bacteriocin-like inhibitory substances (BLISes) (Giraffa et al., 2010). According to some authors (Jack, Tagg & Ray, 1995; Tagg, Dajani & Wannamaker, 1976), the qualified term BLIS is recommended when a newly discovered bacteriocin is not completely characterized regarding its amino acid sequence and the nucleotide sequence of the corresponding structural gene. At this stage, the antimicrobial substance should not be named a bacteriocin.

Typically, the production of antimicrobial compounds by fermentative processes using LAB may be considered somewhat expensive. It is estimated that 30% of the total funds spent on such a process is costs associated with the culture medium and supplements required to promote cell growth of the microorganism producing such molecules (Bali et al., 2014). Reducing the cost of these processes can be highly attractive, especially for small economies

and/or developing countries, where food security can be seriously compromised (Holzapfel, 2002). As an alternative, several agroindustrial waste products rich in nutrients have been used as low-cost substrates instead of synthetic culture media (Panesar & Kaur, 2015).

Cheese whey, the main byproduct of the dairy industry, has high nutritional value and has attracted interest among researchers around the world because of its functional and economic prospects (Koutinas et al., 2009). The high concentration of lactose and the presence of vitamins, minerals, and proteins makes cheese whey an interesting medium for LAB growth and meets the goal of promoting economical production of bioproducts of industrial interest (Smithers, 2015). On the other hand, despite sufficient nutritional composition, cheese whey contains a low concentration of free amino acids and peptides, which are essential for the production of some biocomposites (Vasala, Panula, & Neubauer, 2005). In general, LAB have an inefficient proteolytic system and are highly dependent on external supplements for building of peptides and proteins and even for growth (Pleissner & Venus, 2016; Ummadi & Curic-Bawden, 2010). In this sense, some researchers have proposed enzymatic hydrolysis of the protein fraction of this substrate for increasing the availability of the aforementioned substances and, consequently, increasing the yield of production of biocomposites by different LAB species (Brandelli et al., 2015; Briczinski & Roberts, 2002; Castro & Sato, 2014; Rodríguez-Pazo et al., 2016; Rodríguez-Pazo, Vázquez-Araújo, Pérez-Rodríguez, Cortés-Diéguez, & Domínguez, 2013).

Additionally, in accordance with the trend of economically viable fermentative processes, the use of agricultural byproducts, such as soybean flour, as a protein supplement for hydrolyzed cheese whey, can be an interesting alternative. The benefit of the supplementation of fermentative processes with soybean flour is obvious and is increasingly supported by published evidence. Soybean is one of the most recognized sources of vegetable protein. In addition to proteins, this grain contains basic nutrients such as lipids, vitamins, minerals, sugars, isoflavones, flavonoids, saponins, and peptides (Sanjukta & Rai, 2016). According to Saxena (2015), this substance has already been used as a source of nitrogen to improve commercial production of the antibiotic streptomycin. Moreover, some researchers have reported the use of a soybean protein-based culture medium for production of bacteriocin, demonstrating successful applications, because they attained higher production of this biomolecule when compared with the use of other industrial byproducts (Dominguez, Bizani, Cladera-Olivera & Brandelli, 2007; Motta & Brandelli, 2008; Leães, Vanin, Sant'Anna & Brandelli, 2011). On the other hand, there are no reports in the literature about

the use of soybeans as an alternative source of nitrogen for supplementation of culture media containing zero or low concentration of this nutrient, a fact that adds value to the present study.

In this context, the aim of this work was to study the production of BLIS and lactate by *L. plantarum* ST16Pa cultivated in prehydrolyzed fresh cheese whey as a fermentative substrate. Subsequently, the effect of soybean flour as a protein supplement for the hydrolyzed cheese whey was investigated. Finally, the biopreservative efficiency of cell-free supernatant (CFS), arising from the reproduction of the best culture conditions in a bioreactor, and a solution of *L. plantarum* ST16Pa cells were studied when applied to the surface of chicken breast fillets artificially contaminated with *Enterococcus faecium* 711.

2. Materials and methods

To summarize each step and procedure described in detail below, a flowchart is presented in Figure 5.1.

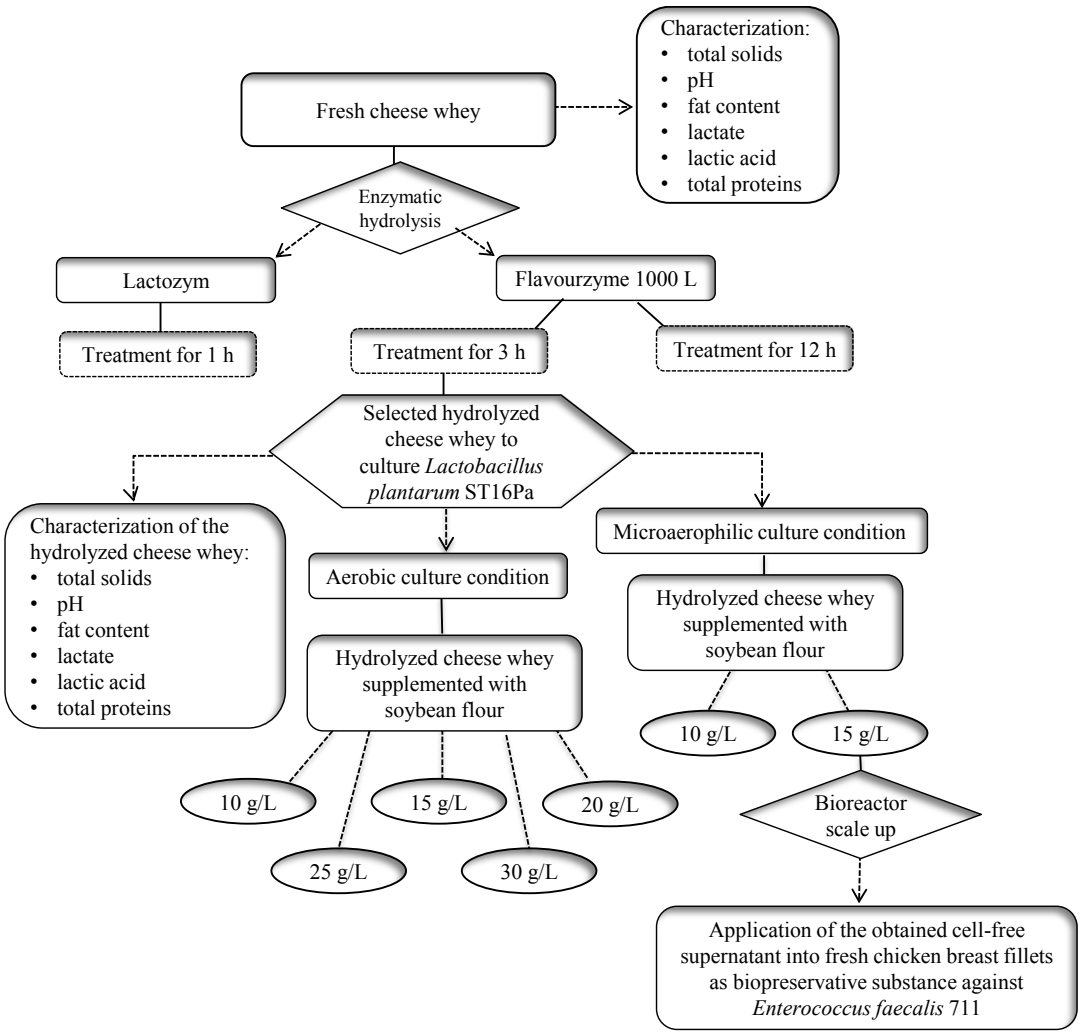

Fig. 5.1. A flowchart summarizing the steps and procedures involved in this study.

2.1. Microbial cultures

For the development of the fermentative processes, probiotic strain *Lactobacillus plantarum* ST16Pa isolated from a papaya species (Todorov et al., 2011) was used. As a microorganism indicative of antimicrobial activity and also as a contaminant of chicken breast fillets, strain *Enterococcus faecium* 711, which was isolated from a sample of food and was provided by the Food Department of the School of Pharmaceutical Sciences of the University of São Paulo (São Paulo, SP, Brazil) was used. Both microorganisms were grown at 30 °C for 24 h in appropriate culture broth (Brain Heart Infusion [BHI] for *E. faecium* 711, and Man, Rogosa, and Sharpe [MRS] broth for *L. plantarum* ST16Pa). The strains were cryopreserved and stored at −70 °C after addition of 20% (v/v) of glycerol to each broth.

2.2. Fresh cheese whey

This whey was kindly provided by Ruta Xacobea SL, a cheese plant located in Brea (A Coruña, Spain), which was fractionated and stored in plastic bottles and finally stored in an ultra-freezer at −70 °C until use.

2.2.1. Characterization of fresh cheese whey

Before use as a culture medium for the cultivation of the *L. plantarum* ST16Pa strain, cheese whey was characterized in order to verify some parameters such as total solids concentration, pH, fat content, and concentrations of lactate, lactose, and proteins.

The determination of the total solids concentration by dry weight was performed using 10 mL of cheese whey, which was transferred to porcelain capsules and incubated in an oven at 105 °C up to constant weight.

The concentration of total protein was determined by means of the Pierce bicinchoninic acid (BCA) assay kit (Thermo Scientific, Aalst, Belgium) using bovine serum albumin as a standard. Via a calibration curve associating albumin concentrations (μg/mL) and respective absorbance (562 nm), an equation ($y = 1.1194x + 0.0378$, $R^2 = 0.99$) was generated, which was used to determine the concentration of total protein present in fresh cheese whey and after hydrolysis.

The pH level of cheese whey was analyzed as described in section 2.6.

High performance liquid chromatography (HPLC) was employed for quantification of sugars and lactate organic acid according to the conditions set forth in section 2.7.

To determine fat content, samples of cheese whey were hydrolyzed with HCl in a system called FOSS Soxtec (2047 SoxCap, Höganäs, Switzerland). After that, 3 g of cheese whey was subjected to liquid-solid extraction in an extractor device using hexane. The fat concentration was calculated by gravimetric difference.

2.2.2. Enzymatic hydrolysis of fresh cheese whey

To increase the production of biocomposites during cultivation of *L. plantarum* ST16Pa with cheese whey as a substrate, enzymatic hydrolysis of the protein fraction was performed. For this purpose, the activities of the enzyme Lactozym Pure 6500 L (β-galactosidase) and of the commercial enzyme mixture Flavourzyme 1000 L (endo and exoprotease activity), both sold by a company called Novozymes (Copenhagen, Denmark), were evaluated.

Hydrolysis with Lactozym was conducted according to the best conditions established by Rodriguez-Colinas, Fernandez-Arrojo, Ballesteros, & Plou (2014). Briefly, 1.20 mL of the enzyme was used to hydrolyze 74.5 g/L total solids. Next, in a metabolic shaker, the mixture was incubated for 1 h at 40 °C/200 rpm.

For enzymatic hydrolysis using Flavourzyme 1000 L, pH of fresh cheese whey was adjusted to 7.0 with 10N NaOH. At the ratio of 0.53 mL to 74.5 g/L of total solids (Brinques et al., 2010), and carrying out the process at 50 °C/100 rpm in a metabolic shaker (Rodríguez-Pazo et al., 2013), we investigated the effectiveness of the enzymatic hydrolysis by Flavourzyme 1000 L for 3 and 12 h.

After such procedures, both hydrolysis reactions were discontinued by the addition of 10 N HCl until pH was between 3.5 and 4.0.

As a control for the performance on the production of antimicrobial biocomposites by *L. plantarum* ST16Pa in the aforementioned procedures, fresh cheese whey without enzymatic hydrolysis was used.

2.2.3. Supplementation of fresh cheese whey

Soybean flour, as a protein supplement, was tested for its potential to enhance the performance of *L. plantarum* ST16Pa, and consequently, to ensure high production yields of BLIS and lactate. To this end, the hydrolyzed cheese whey was supplemented with different

concentrations (10, 15, 20, 25, or 30 g/L) of soybean flour (El Granero integral, Biogran, Austria). Aerobic and microaerophilic culture conditions were also analyzed. Fresh cheese whey hydrolyzed by Flavourzyme 1000 L and without supplementation served as a control.

After supplementation, the hydrolyzed fresh cheese whey was sterilized at 100 °C for 60 min, cooled, and pH was adjusted to 6.5 using 10 N NaOH. Finally, the whey was kept at room temperature until further use.

2.3. Inoculum preparation and culture conditions in a shaker

To prepare the inoculum of *L. plantarum* ST16Pa, 1 mL of the stock culture was transferred to a 250-mL Erlenmeyer flask containing 100 mL of MRS broth (DIFCO, Detroit, MI, USA). Then, it was incubated in a metabolic shaker, under the following culture conditions: 100 rpm/30 °C for 16 h, to achieve a cell concentration of 0.8–0.9 optical density (OD) units at 650 nm per milliliter. Cells were recovered by centrifugation at 3,421 *g* for 15 min (Ortoalresa, Consul 21, EBA 20, Hettich Zentrifugen, Germany). The cell *pellet* was washed with sterile distilled water, centrifuged, and resuspended in fresh cheese whey, already prepared according to the conditions described in subsections 2.2.2 and 2.2.3.

Regarding the cultivation conditions, at first, the fermentation reactions were conducted at the bench scale under aerated and microaerophilic conditions. A 40-mL aliquot (10% [v/v] of the final volume to be fermented) was transferred to 1-L Erlenmeyer flasks containing 360 mL of fresh cheese whey prepared as described in subsections 2.2.2 and 2.2.3 for aerobic cultures conducted in a metabolic shaker (Optic Ivymen System, Comecta SA, Madrid, Spain) at 100 rpm. For microaerophilic conditions, 15-mL tubes were filled with 13.5 mL of fresh cheese whey prepared as described above. A 1.5-mL aliquot (10% [v/v] of the final volume to be fermented) was transferred to the tubes, leaving them completely filled, which were incubated in a microaerophilic jar under static conditions (without stirring). A candle was lit inside the jar to partially reduce the amount of oxygen, leaving no more than 5% of this gas, guaranteeing hypoxia in this system.

All cultures were carried out at 30 °C for 48 h. Samples were periodically collected for viable-cell counting, measurement of lactate and lactose concentrations, and for determination of pH and antimicrobial activity of the CFS. The entire procedure was performed in triplicate.

2.4. Scaling up in a bioreactor

On the basis of the data obtained in the aforementioned tests, scaling up of the fermentation process—that generated the best results with respect to the production of biomass and the BLIS biocomposites and lactate—was carried out.

A mechanical agitation bioreactor (Biostat B plus, Sartorius) with capacity of 2 L was used to conduct this experiment. To this end, 1.35 L of cheese whey, prepared according to the conditions above, was transferred to the tank. After addition of 150 mL of the inoculum (10% [v/v] of the final working volume), nitrogen gas was bubbled through the tank, to establish a concentration of oxygen between 0.4% and 0.5%, guaranteeing an anaerobic process. In triplicate, the cultures were conducted at 30 °C, 150 rpm, for 48 h.

2.5. Cell growth

The monitoring of *L. plantarum* ST16Pa growth was performed by counting viable cells, that is, colony-forming units (CFU)/mL, using the serial dilution technique in which 0.5 mL of the sample was serially diluted in 4.5 mL of 0.85% saline. After that, 100 µL of each dilution was transferred to Petri dishes (90 × 15 mm) containing MRS broth solidified with 1.5% of agar. With the aid of a sterile Drigalski handle, the diluted samples were spread over the surface of the medium plates. The plates were incubated for 48 h and only those containing 20–200 colonies were considered.

To convert the CFU values to cell dry mass (g/L), the *L. plantarum* ST16Pa strain was cultivated in MRS broth under recommended conditions (30 °C/100 rpm), according to Todorov et al. (2011). By associating the CFU values and the corresponding cell dry mass obtained by filtration of the MRS broth through a filtering membrane with 0.22-µm porosity (Millipore, Bedford, MA, USA), a calibration curve was generated, where the obtained equation ($y = 8 \cdot 10^7 x - 1 \cdot 10^7$, $R^2 = 0.98$) served as a basis for conversion of CFU values of the cultures using fresh cheese whey into cell dry mass values in g/L.

2.6. pH monitoring

The samples' pH was measured at predetermined time points using a pH meter (Crisol, Alella, Barcelona, Spain).

2.7. Determination of the concentration of sugars and lactate

To determine the concentration of sugars lactose, glucose, and galactose as well as lactate, samples were aseptically collected and centrifuged at 3,421 *g* for 15 min. The supernatant was filtered through 0.22-µm membranes (Millipore™, Massachusetts, USA), transferred to collection vials, and analyzed on an HPLC system (Agilent, model 1200, USA) according to the conditions set forth by Rodríguez-Pazo et al. (2013).

2.8. Determination of BLIS activity

To detect the antimicrobial activity of the CFS, i.e., BLIS production, samples collected during the fermentation of *L. plantarum* ST16Pa in fresh cheese whey were centrifuged at 3,421 *g* for 15 min. The resulting supernatant was filtered through the membrane with 0.22-µm porosity (Millipore, Bedford, MA, USA) and then tested against bioindicator strain *E. faecium* 711. The test was performed by the agar diffusion method in which the culture broth of the bioindicator strain, precultivated for 16 h in BHI broth, was diluted 100-fold (to approximately 10^6 CFU/mL). One milliliter of this dilution was transferred to a Petri dish (90 × 15 mm) containing 10 mL of melted BHI soft agar (containing 0.75% [w/v] of agar). Next, 20 µL of the CFS was pipetted onto the agar surface. A period of 3 h was allowed for the diffusion of the supernatant at room temperature and after that, the plates were incubated at 30 °C for 24 h. Then, the inhibition halos were measured using a digital caliper and the antimicrobial activity was defined in AUs, which represent the area of the inhibition halos per unit of volume in mL (mm^2/mL), calculated by the procedure adapted from the methodology presented by Sidek et al. (2016).

2.9. BLIS biopreservative efficiency in fresh chicken breast fillets

To investigate the biopreservative efficiency of CFS against the growth of bioindicator strain *E. faecium* 711, fresh chicken breast fillets (at 24 h *post mortem* and individually aged on ice) were purchased in a local market, and transported to the laboratory in a refrigerated thermal box (~4 °C). The protective capacity of strain *L. plantarum* ST16Pa was also evaluated.

The chicken fillets were aseptically cut into pieces of approximately 7 × 7 cm and weighing 25 g. After that, the microbial load of possible contaminants was reduced using UV rays for 15 min on each surface.

Five series of experiments were conducted in this study. As a first step, in tests A, B, C, and D, each surface of the chicken fillets was artificially contaminated with 500 µL of *E. faecium* 711, which was precultivated in BHI broth at 30 °C for 16 h and then diluted 400-fold in 0.85% sterile saline (final concentration 10^3 CFU/mL). Next, individually, the chicken fillets were treated as described below. In test A, 1 mL of CFS was pipetted and evenly spread with the aid of a sterile Drigalski handle on each surface of the fillets. After that, ten minutes were allowed for the disappearance of the excess of fluid in the fillets.

In test B, a solution of *L. plantarum* ST16Pa cells was applied to the surface of the fillets to verify the protective capacity of this strain. For this purpose, *L. plantarum* ST16Pa was cultivated in MRS broth at 30 °C for 16 h (~10^7 CFU/mL). It was centrifuged at 3,421 *g* for 15 min, and the resulting cell *pellet* was resuspended in 0.85% sterile saline. One milliliter of this solution was pipetted onto the chicken fillets following the same protocol as described above.

Tests C, D, and E served as controls. Thus, to monitor the growth of the bioindicator strain, in test C, the chicken fillets were artificially contaminated, but no biopreservative substances were added. In the test D, the pure commercial pediocin (Sigma–Aldrich, St. Louis, MO, USA) served as the control of the antimicrobial action against *E. faecium* 711 on fresh chicken fillets, because it is a pure and well-known substance. To this end, starting from an initial known concentration (0.1 mg/mL), different dilutions of the pure commercial pediocin were prepared in 0.1 M sodium acetate buffer (pH 5.0). To verify the size of the inhibition halos generated by each dilution, they were deposited on the solid surface of the BHI soft agar preinoculated with 10% (v/v) of the bioindicator strain (~10^7 CFU/mL). By associating the corresponding concentration (µg/mL) with the generated inhibition halos (mm), a calibration curve ($y = 2.7412x + 11.714$, $R^2 = 0.99$) was obtained, which was useful for determining the concentration of pediocin that would be used to treat chicken fillets. The identified concentration (0.71 µg/mL) corresponded to the size of the inhibition halo formed by CFS (14.34 mm). One milliliter of this solution was applied to the surface of the chicken fillets according to the procedure described above. Finally, to verify whether the chicken fillets were not contaminated by another *Enterococcus* strain, in test E, the chicken fillets were not artificially contaminated by the bioindicator strain and were not treated with any biopreservative substance.

All chicken fillets from tests A, B, C, D, and E were packed in sterile plastic bags and stored in an unchanged atmosphere in a refrigerator at 4 ± 0.5 °C. The tests were conducted in triplicate, and the samples, for counting the bioindicator strain cells in CFUs per gram of fillet (CFU/g), were collected immediately after the inoculum (day 0), during 3 consecutive days (days 1, 2, and 3) and after 7 and 14 days.

To monitor the growth of *E. faecium* 711 during the storage, 225 mL of 0.85% sterile saline was added to the plastic bags containing the chicken fillets. It was homogenized for 2 min in a *Stomacher* device (IUL Instruments, Barcelona, Spain), and the resulting product was serially diluted 10-fold as described in section 2.5. Ten microliters of each dilution was deposited on the surface of the Slanetz Bartley agar culture medium (Liofilchem Diagnostici, Italy) supplemented with 1% (w/v) of the solution of 2,3,5-triphenyltetrazolium chloride (TTC) (Scharlau Microbiology, Barcelona, Spain). It is a selective medium for the genus *Enterococcus* and inhibits the growth of Gram-negative bacteria because of the presence of sodium azide. The genus *Enterococcus* reduces the TTC compound, producing a purple precipitate that gives reddish color to their colonies.

Finally, the plates were incubated at 30 °C, and after 72 h, the CFUs were counted. The serial dilution was considered valid when it was possible to count from 3 to 30 CFUs.

2.10. Statistical analysis

All the data were subjected to analysis of variance (ANOVA) in statistical *software* Minitab 17 (Inc., State College, PA, USA). Data were also compared by the Tukey test ($P < 0.05$), and different letters were used to indicate values with significant differences between them.

3. RESULTS AND DISCUSSION

3.1. Enzymatic hydrolysis and characterization of fresh cheese whey

The results on biomass and BLIS and lactate production, generated by cultivating *L. plantarum* ST16Pa in fresh cheese whey hydrolyzed by each of the mentioned enzymes, are presented in Table 5.1.

Table 5.1. Production of biomass and lactate and BLIS activity after culturing *L. plantarum* ST16Pa for 48 h in raw fresh cheese whey or that hydrolyzed by Lactozym or Flavourzyme

Fresh cheese whey	Biomass (g/L)	Lactate (g/L)	BLIS activity (AU/mL)
Raw	0.33±0.04[a]	1.85±0.15[a]	-
Lactozym	0.40±0.04[a]	3.00±0.28[b]	4,204.66±57.45[a]
Flavourzyme 3 h	1.73±0.53[c]	6.67±0.10[d]	5,058.64±314.87[b]
Flavourzyme 12 h	0.73±0.02[b]	6.18±0.26[c]	4,369.52±175.69[a]

In raw fresh cheese whey, *L. plantarum* ST16Pa generated 0.33 ± 0.04 g/L biomass, 1.85 ± 0.15 g/L lactate, and did not show formation of inhibition halos against the bioindicator strain *E. faecium* 711.

In particular, Lactozym showed 75% efficiency of the conversion of lactose (initial concentration 54 g/L) into glucose (20 g/L after hydrolysis) and galactose (20.8 g/L after hydrolysis). According to Geiger et al. (2016) and Panesar et al. (2006), this enzyme can perform transgalactosylation reactions, which are gaining interest due to the formation of galacto-oligosaccharide (GOS) molecules, known to be a prebiotic ingredient that favors the growth of probiotic microorganisms, such as *L. plantarum* ST16Pa (Todorov et al., 2012). In fact, after 48 h of culture, galactose was completely metabolized, while glucose by only 10% (w/v). After the hydrolysis of fresh cheese whey by Lactozym, the results on biomass (0.40 ± 0.04 g/L), BLIS activity (4,204.66 ± 57.45 AU/mL), and lactate production (3.00 ± 0.28 g/L) by *L. plantarum* ST16Pa were considerably higher when compared to the performance in raw cheese whey.

On the other hand, the 3-h hydrolysis treatment with Flavourzyme showed considerable differences, increasing the biomass production (1.73 ± 0.53 g/L) and BLIS activity (5,058.64 ± 314.87 AU/mL), which are 2.37- and 1.16-fold higher, respectively, relative to the data generated by hydrolysis with Flavourzyme for 12 h, and 4.32- and 1.20-fold higher, respectively, compared to the data obtained after fermentation in Lactozym-hydrolyzed cheese whey. Rodríguez-Pazo et al. (2016) and Sinha, Radha, Prakash, & Kaul (2007) reported that this enzyme acts as endo- and exopeptidase, and during its hydrolysis, proteins are fragmented into free amino acids and peptides of different sizes. Consequently, this process increases the concentration of nitrogen sources, which are essential for the growth of microorganisms and favor cellular performance and, consequently, the productivity in terms of biocomposites (Castro & Sato, 2014).

Accordingly, it was found that the best pretreatment of fresh cheese whey for the cultivation of *L. plantarum* ST16Pa and for a satisfactory yield of biomass and antimicrobial

bioproducts was 3-h hydrolysis with Flavourzyme 1000 L. In fact, longer periods of hydrolysis, such as 12 h, can cleave some proteins that are essential for the growth, thus preventing the strain from developing as expected. Therefore, the treatment of cheese whey for a shorter period is considered a favorable situation, not only from the standpoint of productivity but also from the economic point of view, given that 12-h hydrolysis results in a loss of investment and becomes unfeasible to industries.

Regarding the results obtained with Lactozym-hydrolyzed cheese whey, they suggest that the performance of *L. plantarum* ST16Pa depends not only on easily absorbed carbon sources but also on essential amino acids and peptides.

After identifying the best hydrolysis method, we decided to carry out the cheese whey characterization before and after this treatment. In particular, except for total solids (from 74.5 ± 0.7 to 47.7 ± 0.14 g/L) and proteins (from 7.3 ± 0.14 to 5.9 ± 0.28 g/L), the other parameters did not show significant differences between the composition of the raw and hydrolyzed cheese whey (pH varying from 6.45 ± 0.07 to 6.8 ± 0.28; lactose from 54.15 ± 0.23 to 54.10 ± 0.16 g/L; lactate from 0.12 ± 0.01 to 0.20 ± 0.07 g/L, and the same value of 3.70 ± 0.14 g/L for fat content). The fact that the hydrolysis of cheese whey promotes fragmentation of its proteins into smaller polypeptides, which have more binding sites for water molecules, increases the solubility of this substrate (Corrêa et al., 2014; Rodríguez-pazo et al., 2016; Sinha et al., 2007). This observation can explain the reduction in total solids content from 74.5 to 47.7 g/L and total protein from 7.3 to 5.9 g/L. In addition, the decrease in the total protein amount by approximately 20% ensures that the hydrolysis with Flavourzyme exerted the activity expected from this enzyme.

3.2. The fermentation process

As previously mentioned, cheese whey, despite nutritional support for cultivation of microorganisms, especially LAB, may have a low concentration of free amino acids and peptides, which are essential for the production of certain biocomposites (Vasala et al., 2005). This finding, supposedly, justifies the performance of *L. plantarum* ST16Pa in the work of Todorov et al. (2011), who did not observe production of bacteriocins when this microbe was cultivated in cheese whey, despite a reasonable rate of cell growth.

According to Guerra and Pastrana (2001), it is possible to turn cheese whey into a suitable culture medium for the production of bacteriocins through supplementation with nitrogen sources. Thus, on the basis of the results presented in the previous section, in order to

improve the performance on BLIS and lactate production by *L. plantarum* ST16Pa in fresh cheese whey hydrolyzed by Flavourzyme 1000 L for 3 h, this substrate was supplemented with soybean flour at concentrations of 10, 15, 20, 25, or 30 g/L under aerobic conditions and at 10 or 15 g/L in microaerophilia. In fact, concentrations higher than 15 g/L could not be tested under microaerophilic conditions because soybean flour has insoluble fiber in its composition (19%), which collects at the bottom of the tubes used for these cultures, making it difficult to distribute and homogenize the nutrients.

Containing 41% of proteins, the soybean flour used in this study was shown to be an appealing organic source of nitrogen for supplementation of hydrolyzed cheese whey and, subsequently, to be used as a substrate for the cultivation of *L. plantarum* ST16Pa, with the aim to produce BLIS and lactate.

Table 5.2 summarizes the biomass data, lactate production, BLIS activity, as well as the fermentative parameters calculated from the data generated by the above-mentioned tests. It was found that supplementation with soybean flour significantly improved the performance of *L. plantarum* ST16Pa. Under microaerophilic conditions, when control A is compared with the test involving 10 g/L soybean flour, an increase in the cellular concentration (0.94 ± 0.01 vs. 2.46 ± 0.19 g/L), BLIS activity (5,191.21 ± 124.67 vs. 7,367.23 ± 152.09 AU/mL), and lactate (7.98 ± 0.12 vs. 13.84 ± 0.46 g/L) was observed. Zhang et al. (2014) also reported that the addition of 10 g/L soybean flour was sufficient to increase the microbiological counts of LAB species.

Under the aerobic conditions, the addition of 15 g/L soybean flour to the hydrolyzed cheese whey gave the best results in terms of biomass concentration (5.01 ± 0.08 g/L) and conversion of lactose into biomass ($Y_{biomass/lactose}$ = 0.40 ± 0.01 g/g). On the other hand, we noticed that the supplementation with soybean flour at 20 g/L under aerobic conditions and 15 g/L under microaerophilic conditions decreased the performance of *L. plantarum* ST16Pa, probably due to catabolite repression (Abdel-Rahman et al., 2015; Zotta et al., 2012).

In addition, a higher yield (28%) of lactose conversion into lactate was observed ($Y_{lactate/lactose}$ = 1.39 ± 0.04 g/g) under the microaerophilic conditions with hydrolyzed cheese whey supplemented with 10 g/L soybean flour, in comparison to aerobic conditions with hydrolyzed cheese whey supplemented with 15 g/L soybean flour ($Y_{lactate/lactose}$ = 1.08 ± 0.01 g/g).

On the basis of the obtained results, it was observed that both the microaerophilic culture and the supplementation with 10 g/L soybean flour gave an additional economic advantage to the fermentation process, justifying the choice of those conditions for reproduction of the process on a pilot scale using a mechanical agitation bioreactor.

Table 5.2. Fermentative parameters, biomass production, lactate, and BLIS activity of L. plantarum ST16Pa grown in a shaker or bioreactor

	Shaken cultivation									Bioreactor
	Microaerophilia			Anaerobiosis						
Soybean flour supplementation (g/L)	**0 (control A)**	**10g/L**	**15g/L**	**0 (control B)**	**10g/L**	**15g/L**	**20g/L**	**25g/L**	**30g/L**	**10g/L**
Biomass (g/L)	0.94±0.01^{a}	2.46±0.19^{d}	1.90±0.08^{c}	1.72±0.54^{b}	3.65±0.80^{ef}	5.01±0.08^{h}	4.64±0.10^{g}	4.21±0.18^{f}	4.07±0.17^{f}	3.14±0.06^{e}
BLIS activity (AU/mL)	5,191.21±124.67^{d}	7,367.23±152.09^{e}	5,746.99±134.33^{e}	4,044.52±169.02^{a}	4,895.16±40.11^{c}	5,191.21±127.67^{d}	4,793.41±184.01^{c}	4,410.52±117.68^{b}	4,452.72±177.35^{b}	8,082.56±157.64^{f}
Lactate (g/L)	7.98±0.12^{b}	13.84±0.46^{e}	12.64±0.07^{d}	6.69±0.13^{a}	10.63±0.10^{c}	10.76±0.16^{c}	11.19±0.47^{c}	13.53±0.45^{e}	13.53±0.18^{e}	14.33±0.03^{f}
μ_{max} (h^{-1})	0.23±0,01^{a}	0.38±0.04^{b}	0.39±0.00^{b}	0.35±0.04^{b}	0.42±0.03^{b}	0.40±0.02^{b}	0.40±0.01^{b}	0.41±0.01^{b}	0.43±0.02^{b}	0.35±0.01^{b}
T_g (h)	3.02±0.19^{b}	1.86±0.18^{a}	1.77±0.00^{a}	2.02±0.21^{a}	1.65±0.11^{a}	1.67±0.08^{a}	1.73±0.06^{a}	1.71±0.03^{a}	1.63±0.08^{a}	1.98±0.08^{a}
$Q_{lactate}$ (g/L.h)	0.17±0.01^{b}	0.29±0.01^{e}	0.26±0.00^{d}	0.14±0.00^{a}	0.22±0.00^{c}	0.23±0.01^{c}	0.23±0.01^{c}	0.28±0.01^{d}	0.28±0.00^{d}	0.30±0.00^{f}
$Q_{biomass}$ (g/L.h)	0.04±0.00^{a}	0.11±0.01^{c}	0.08±0.00^{b}	0.07±0.03^{abc}	0.15±0.03^{d}	0.21±0.01^{f}	0.19±0.00^{e}	0.18±0.01^{e}	0.17±0.01^{e}	0.13±0.00^{d}
$Y_{biomass/lactose}$ (g/g)	0.13±0.00^{a}	0.25±0.02^{c}	0.16±0.01^{b}	0.24±0.08^{c}	0.37±0.06^{efg}	0.40±0.01^{g}	0.32±0.01^{cd}	0.35±0.01^{ef}	0.33±0.01^{d}	0.30±0.00^{c}
$Y_{lactate\ /lactose}$ (g/g)	1.09±0.02^{a}	1.39±0.04^{a}	1.05±0.03^{a}	0.93±0.02^{a}	1.08±0.01^{a}	1.05±0.01^{a}	1.03±0.04^{a}	1.04±0.04^{a}	0.98±0.01^{a}	1.37±0.00^{a}

μ_{max}: maximum specific growth rate; T_g: generation time; $Y_{biomass/lactose}$: Lactose to biomass yield; $Y_{lactate/lactose}$: Lactose to lactate yield; $Q_{biomass}$: global volumetric productivity of biomass; $Q_{lactate}$: global volumetric productivity of lactate (48 h cultivation).

3.3. Bioreactor cultivation

The values of biomass, BLIS activity, lactate concentration, as well as the fermentative parameters of the assay developed in the bioreactor are presented in Table 2.

The agitation at 150 rpm and the fermentation conducted during complete anaerobiosis may have favored the distribution of the nutrients present in the culture medium, thus yielding better performance, especially in terms of cell growth (3.14 ± g/L) and BLIS activity (8,082.56 ± 157.64 AU/mL) when compared to the results obtained in the shaker.

3.4. Biopreservation of fresh chicken breast fillets

The growth of the bioindicator strain on the chicken fillets was followed for 14 days, and the results of the treatments with the CFS (test A) and the cell producer solution (test B) were compared with the control treatments: fillets without addition of any preservative substance (test C) and with pure commercial pediocin (test D).

In Figure 5.2, the growth of bioindicator *E. faecium* 711 in each of the treatment groups used to extend the shelf life of chicken fillets is shown. The results presented are related to tests A, B, C, and D because test E served only as a control of fillet sterility, not showing the growth of other strains of genus *Enterococcus*.

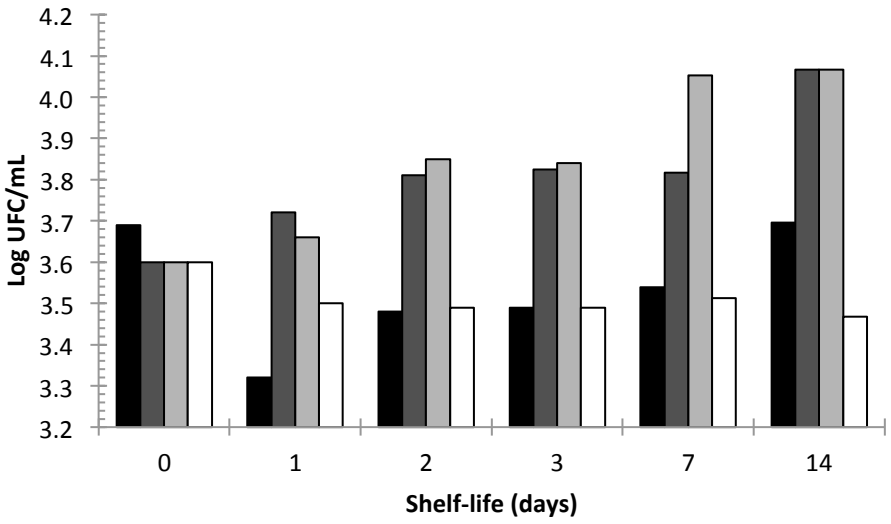

Fig. 5.2. Growth of *Enterococcus faecium* 711 in artificially contaminated fresh chicken breast fillets according to experimental tests: A (treated with the cell-free supernatant, ■); B: (treated with *L. plantarum* ST16Pa cells, ■); C: (without biopreservative substances, ■); and D: (treated with commercial pediocin, □).

It was found that the chicken fillets not treated with any preservative substance (test C) showed considerable growth of the bioindicator strain at 4.07 log CFU/g after 7 days of storage under refrigeration (4 ± 0.5 °C). During treatment with CFS (test A), with the *L. plantarum* ST16Pa cell solution (test B), or pediocin (test D), the growth of the bioindicator strain was 3.54, 3.82, and 3.51 log CFU/g, respectively, indicating that all treatments are effective at controlling the contaminant strain.

For each treatment used, it was found that application of pure pediocin to chicken fillets (test D) was the most effective, being able to completely inhibit the growth of the bioindicator strain during the whole storage period (3.47 log CFU/g at the end of 14 days of storage). The effectiveness of treatment with pediocin was already expected and was corroborated by several researchers, who verified its *ex situ* efficiency by applying it to a wide variety of meats, against numerous contaminant species and foodborne pathogens (Chen & Hoover, 2003; Espitia, Pacheco, Melo, Soares, & Durango, 2013; Goff, Bhunia, & Johnson, 1996; Ming, Weber, Ayres, & Sandine, 1997; Santiago-Silva et al., 2009; Woraprayote et al., 2013).

For the treatment of the chicken fillets with CFS (Test A), it was observed that bioindicator strain *E. faecium* 711 was completely inhibited until the 7th day of storage (3.54 log CFU/g). Even so, from day 7, the supernatant continued to show reduced antimicrobial activity (a 21% decrease in effectiveness as compared to test C).

The reduction of antimicrobial effectiveness may be associated with the instability of the bacteriocin molecule when applied in food. Among the several factors limiting the effectiveness of *ex situ* bacteriocins, are those mainly related to the food in question, such as inactivation by proteolytic enzymes, oxidative processes, adsorption of bacteriocin molecules to food components (proteins present on the food surface and/or fat particles), and low solubility and/or inadequate distribution in the food (Gálvez, Abriouel, López, & Omar, 2007). The ways to overcome these obstacles may be application of the purified or prepurified compound to food; encapsulation of the compound by microencapsulation technologies; or direct inoculation into the food with the bacteriocin-producing strain (Schillinger et al., 1996).

During the first 3 days of storage, test B was not effective and no antimicrobial activity against the bioindicator strain was observed. Nonetheless, after this period, the treatment in question was shown to be effective, controlling the growth of *E. faecium* 711 at 3.82 log CFU/g, 48% lower than in test C. In contrast, the effectiveness of this treatment was 45% lower than that of the treatment with CFS (test A) during the same period of time.

In similar studies described in the literature (Anacarso et al., 2014; Katla et al., 2002; Rivas, Castro, Vallejo, Marguet, & Campos, 2014), it is reported that application of a cell solution of certain bacteriocin-producing microorganisms to the meat surface is much more effective when compared to the antimicrobial action of bacteriocin itself (*in situ* vs. *ex situ* activity, respectively).

The antimicrobial ineffectiveness observed in test B (the solution of *L. plantarum* ST16Pa cells against *E. faecium* 711) in the early days can be explained by the fact that both bacteriocin and lactate are produced at higher concentrations in the late stationary growth phase of *L. plantarum* ST16Pa. Both compounds are mainly responsible for the antimicrobial activity expressed by LAB (Leroy & De Vuyst, 2004). Considering that the conditions established in this test did not favor the growth of *L. plantarum* ST16Pa, mainly owing to the selected low temperature (4 ± 0.5 °C), it is understandable and reasonable that it exerted antimicrobial activity against the bioindicator strain only after the third day of storage of chicken fillets.

In summary, among all the treatments used in this work to prevent the contamination and deterioration of fresh chicken fillets by microorganisms belonging to the genus *Enterococcus*, it was found that test D was the most effective, exerting biopreservative activity during 14 days of storage. However, commercial pediocin has a very high cost due to its high purity (≥95%), which makes its application economically unfeasible. On the other hand, the results obtained in test A showed that CFS was effective and a promising alternative for control of contamination by *E. faecium* 711.

4. Conclusions

Hydrolyzed cheese whey was shown to be a culture medium with a great potential for the cultivation of *L. plantarum* ST16Pa strain and consequent production of BLIS and lactate. Soybean flour proved to be an alternative source of protein for supplementation of cheese whey. In chicken breast fillets contaminated with *E. faecium* 711, CFS showed high antimicrobial efficiency during 7 days of refrigerated storage, but it is necessary to test its antimicrobial activity against different foodborne pathogens in the future. In particular, the use of CFS may give an additional advantage from the economic point of view because to exert the biopreservative efficiency similar to that of pure commercial pediocin, no purification steps were necessary (these step may raise the cost of biotechnological production). Moreover, the fact that CFS was obtained from the reuse of cheese whey—the main waste of the cheese industry—indicates an ecofriendly approach.

FINAL CONCLUSIONS AND PROSPECTS

The studies developed in the chapters previously presented in this thesis demonstrate that *L. plantarum* ST16Pa is a good bacteriocin-producing strain, which can grow and produce bacteriocin not only in the recommended culture medium (MRS) but also in an industrial byproducts, such as whey – fresh or powder – since adequately supplemented with an additional nitrogen source. Besides, *L. plantarum* ST16Pa demonstrated to be quite adaptable to different culture conditions that was submitted, but preferentially producing higher bacteriocin activity under restricted oxygen conditions (stationary, microaerophilic or anaerobic conditions). No more than that, the antimicrobial activity of CFS obtained by culturing *L. plantarum* ST16Pa in hydrolyzed fresh cheese whey showed high biopreservative efficiency in chicken breast fillets artificially contaminated with a food pathogen, indicating its potential to be used as a biopreservative in the food industry.

Regarding the purification of the bacteriocin produced by *L. plantarum* ST16Pa, the proposed protocols attained satisfactory recovery results, however, were not sufficiently suitable to obtain a pure molecule. Considering that the devices for determination of molecular weight and amino acid sequences (for example, mass spectrometry and Edman degradation, respectively) require an ultrapure bacteriocin, this step could not be performed at moment, but will be investigated in a future work by our research group.

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APPENDIX A – Article in collaboration submitted to *International Journal of Food Microbiology* (impact fator: 3.44).

Evaluation of brewer's spent grain to formulate culture media for the production of bacteriocins by Patagonian strains

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Abstract

The study was conducted to evaluate the production of bacteriocins by culturing 17 Patagonian strains with antimicrobial activity against spoilage and pathogens microorganisms. *Lactococcus lactis* Tw11 and *Enterococcus mundtii* Tw492 were selected to grow in brewery spent grain hemicellulosic hydrolyzates. The presence of Tween 80 allowed the release of bacteriocins produced by *L. lactis* Tw11 and enhanced 3.4-fold times the amount of bacteriocins generated by *E. mundtii* Tw492, with inhibition halos of 15.46 mm ± 0.050 (after 28h) and 24.47 mm ± 0.090 (after 7h), respectively, against 10^5 cells of *L. monocytogenes*. Additionally, under these conditions, the concentration of lactic acid also increased up to 3.2 g L^{-1} ± 0.121 using *L. lactis* Tw11 and remained similar (2.45 g L^{-1} ± 0.014) with *E. mundtii* Tw492 at 28h. Further supplementation with salts was detrimental probably due to hemicellulosic hydrolyzates were rich culture media.

Keywords: brewery spent grain, hydrolytic treatments, bacteriocins, *Lactococcus lactis, Enterococcus mundtii, Listeria monocytogenes*

1. Introduction

The European Union has produced around 383 million hectoliters of a wide variety of beers in the year 2014, from the 6500 existing breweries, becoming the second largest beer producer in the world after China (Berkhout et al., 2016). During the brewing process, the malted barley is subjected to enzymatic degradation, which releases fermentable (maltose and maltotriosa) and non-fermentable (dextrins) carbohydrates, soluble proteins, polypeptides and amino acids. This medium (named wort) is fermented into beer, meanwhile the insoluble grain components is called the brewery spent grains (BSG) (Fărcaş et al., 2015; Lynch et al., 2016; Russ et al., 2005). BSG represents about 85% of the solid waste produced during the brewing, meanwhile the rest are hops, trub, yeast and a minimal amount of sludge (Lynch et al., 2016; Mussatto, 2014; Olajire, 2012). BSG comprises husks, pericarp and seed coat layers of the barley malt, and in some beers also by rest of adjuncts (corn, rice, wheat, oats, rye or sorghum) added in mixture with the malt for the worth elaboration. The worldwide annual production of BSG has been estimated as approximately 38.6×10^{6} t. BSG is a lignocellulosic material, mainly constituted by sugars, proteins and minerals (Lynch et al., 2016; Mussatto, 2014; Olajire, 2012), although the composition varies depending on the brewing processes (malting and mashing), the stage of harvest, the type of cereal and the adjuncts employed (Santos et al., 2003).

Among the different applications, BSG outstands as a very advantageous feed, rich in protein, fiber and energy, containing 20-25% dry matter, which usual destination is for animal nutrition, either in wet or dried form (Hejna et al., 2015; Lynch et al., 2016; Mussatto, 2014). However, BSG spoils within three days due to its high moisture, polysaccharides and protein content and therefore cannot be long-term stored and consequently, this use has been steadily decreasing (Lynch et al., 2016; Russ et al., 2005). BSG also finds applications on human alimentation based on its health benefits. For

example the protein fraction has interesting properties due to the high level of essential amino acids and as antioxidant (Niemi et al., 2013; Vieira et al., 2017). BSG can also be used for energy production, for the production of charcoal with high calorific value; the production of paper; as a brick component; and as an adsorbent for volatile organic compounds and heavy metals in contaminated waters (Hendriks and Zeeman, 2009; Kumar et al., 2009; Mussatto, 2014; Mussatto et al., 2006; Russ et al., 2005).

On the other hand, the cell walls of this lignocellulosic material, can be degraded into their constituents by hydrolytic processes, such as glucose from cellulose; and xylose, mannose, galactose, arabinose, hydroxycinnamic and acetic acids from non-cellulosic polysaccharides (Mussatto, 2014; Niemi et al., 2012). Although a wide variety of hydrolytic procedures can be found in literature, not all of them are suitable, since the liberated sugars can be degraded under harsher conditions, with the consequent formation of undesirable compounds and inhibitors, such as furfural and hydroxymethylfurfural (Jönsson and Martín, 2016; Mussatto and Roberto, 2006; Palmqvist and Hahn-Hägerdal, 2000; Pérez et al., 2002). One of the most common treatments consists in the realization of a pre-treatment based on an acid hydrolysis (prehydrolysis) that allows solubilizing the hemicellulosic fraction in its constituent monomers (Mussatto et al., 2007; Mussatto and Roberto, 2005). Therefore, the sugars released could be employed as precursor of other add-value compounds or enzymes by microbial transformation (Mussatto, 2014).

However, in our knowledge, no studies have been conducted in the generation of bacteriocins from this material. Bacteriocins are commonly denoted as ribosomally synthesized antimicrobial peptides that usually show specificity against bacteria closely related. The most studied bacteriocins are those produced by lactic acid bacteria (LAB), since they are microorganisms considered as GRAS, but also includes the enterococci. This genus is beginning to be considered as an emerging human pathogen, despite the fact that

several species can be found in our gastrointestinal tract, showing positive effects in food fermentation and preservation (Ness et al., 2014; Rivas et al., 2012). There are five groups of bacteriocins classified according to molecular mass, post-translational modification, presence of modified amino acids, chemical structure and mode of action. In recent years, the interest for these peptides has constantly increased due to their antimicrobial potential in fields of health, agriculture and food preservation (Ahmad et al., 2016; Balciunas et al., 2013; Woraprayote et al., 2016)

This work deals with a primary screening of Patagonian strains, the study of brewery-spent grains (BSG) as feedstock for the generation of hemicellulosic hydrolyzates, and further production of bacteriocins by selected microoganisms. The antimicrobial activity was tested against *Listeria monocytogenes* CECT-934 and *Enterococcus faecium* 101, 104 and 711. The influence of temperature, Tween 80 and salts addition was also considered.

2. Materials and Methods

2.1. Raw material

Two types of Brewer's spent grains (BSG) were kindly provided by Hijos de Rivera Inversiones Corporativas S.L. (La Coruña, Spain). The main differences between samples were variances during the malting and cooking processes of the raw materials, and the percentage of malt attachments. BSG was dried in the laboratory in an oven (Celsius 2007, Memmert, Schwabach, Germany) at 50°C for approximately 48h to prevent microbial contamination during storage. Each BSG was homogenized in a single lot and stored for experimentation at 4°C.

2.2. Characterization of raw material

BSG was oven-dried (Binder-Model 53 ED, Tuttlingen, Germany) to constant weight at 105°C in order to determine the percentage of humidity. The ash content was measured

130 using a muffle furnace (Carbolite ELF 11/6B with 301 controller, Derbyshire, United
131 Kingdom) during 6h at 550°C. Nitrogen and carbon percentages were analyzed using a
132 Thermo Finningan Flash Elemental Analyzer 1112 series, San Jose, CA (USA). The
133 composition of BSG was determined by quantitative acid hydrolysis in two-stages (Pérez-
134 Bibbins et al., 2013). Lignocellulosic material was treated 1h with 72 wt % sulfuric acid at
135 30 °C, then sulfuric acid was diluted until 3 wt% and heated at 121°C during 1h. The solid
136 residue obtained after hydrolysis was oven-dried at 105°C and considered as Klason lignin.
137 The analysis of the liquid fraction by High Performance Liquid Cromatography (HPLC)
138 was described below.
139 The following minerals K, Cu, Fe, Mn, Mg, Ca, and Zn were measured in an Atomic
140 Absorption Spectrometer Varian SpectrAA 220 Fast Sequential (Varian Inc., Palo Alto,
141 CA, EE.UU) by wet digestion with HNO_3. The metallic elements Cr, Co, Ni, Cd, Pb, and
142 Al were determined using an ICP-MS Thermo Elemental X7 (Waltham, Massachusetts,
143 USA).
144 All parameters were performed by triplicate and standard deviations reported in the text.

145 **2.3. Hydrolytic treatment (prehydrolysis)**

146 Acid hydrolysis reactions were performed under two different operational conditions
147 proposed by Mussatto and Roberto (2006, 2005). The first procedure (prehydrolysis 1)
148 treated bagasse during 17 min at 120°C in a liquid/solid ratio of 8 g/g with 100 mg
149 H_2SO_4/g dry matter, meanwhile in the second methodology (denoted as prehydrolysis 2)
150 the bagasse was treated under harsher conditions in a liquid/solid ratio of 10 g/g with 120
151 mg H_2SO_4/g dry matter at 120°C for 27 min.
152 After hydrolysis, pH was neutralized with NaOH pellets, and the solid material was
153 separated using a 100% polyester cloth. The liquid stream was analyzed by HPLC to
154 determine the concentration of released sugars, phenolic compounds and/or furfural. The

155 remaining solid was dried, and characterized by quantitative acid hydrolysis, as previously
156 described. Hydrolyses were performed by duplicate and standard deviations reported in the
157 text.

158 **2.4. Microorganism and culture media**

159 Seventeen strains belonging to genera *Enterococcus, Lactococcus* and *Pediococcus*
160 were examined in this work. All strains, isolated from the Southern Argentine Province of
161 Chubut, were summarized in **Table 1** including their origin. Gut microbiota of marine
162 specimens were collected following the methodology of Sequeiros et al., (2010)
163 meanwhile isolates from Ewes'milk and cheese were obtained using the methods proposed
164 by Rivas et al., (2012). Fecal samples of birds and subsequent microbiota isolation were
165 done following the methodology compiled in Ledesma et al., (2015). *Lactococcus lactis*
166 subsp. *lactis* Tw11 and *Enterococcus mundtii* Tw 492 also were identified through partial
167 16S rRNA gene sequencing. Data sequences were deposited in Genbank under accession
168 number JQ724544 and KY689398, respectively.

169 *Listeria monocytogenes* CECT-934 obtained from the Spanish Collection of Type
170 Cultures (Valencia, Spain) and *Enterococcus faecium* 101, 104 and 711 provided by the
171 Department of Food, Faculty of Pharmaceutical Sciences, University of São Paulo (São
172 Paulo, SP, Brazil), were used as bioindicator microorganisms to evaluate the antimicrobial
173 activity of cell-free extracts obtained after fermentation of the Patagonian strains.

174 All strains were maintained in cryovials at -80 °C using 15% glycerol as cryoprotector.

175 LAB were grown in Man-Rogosa-Sharpe (MRS) broth containing 10 g L^{-1} peptone, 8 g
176 L^{-1} beef extract, 4 g L^{-1} yeast extract, 20 g L^{-1} D(+)-glucose, 2 g L^{-1} K_2HPO_4, 2 g L^{-1}
177 diammonium hydrogen citrate, 5 g L^{-1} CH_3COONa, 0.2 g L^{-1} $MgSO_4.7H_2O$, 0.05 g L^{-1}
178 $MnSO_4.2H_2O$ and 1 g L^{-1} Tween-80. *Listeria monocytogenes* CECT-934 was cultured in
179 Brain Heart Infusion (BHI) broth formulated with peptone mixture 10 g L^{-1}, beef heart

180 infusion 10 g L^{-1}, calf brain infusion 7.5 g L^{-1}, NaCl 5 g L^{-1}, HNa_2PO_4 2.5 g L^{-1} and
181 dextrose 2 g L^{-1}.
182 Commercial media were sterilized in autoclave (Trade Raypa SL, Terrassa, Barcelona)
183 at 121°C for 15 min.

184 **2.5. Screening of bacteriocin-producers**

185 **2.5.1. Production of bacteriocins**

186 Patagonian strains were carried out growing 12h the strains in MRS broth at 30°C and
187 100 rpm in an orbital shaker (Optic Ivymen System, Comecta S.A., distributed by
188 Scharlab, Madrid, Spain). The cells were recollected by centrifugation, washed 3 times
189 with phosphate-buffered saline (PBS: 10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with
190 pH adjusted to 7.0), resuspended in the same buffer and used as inoculum at 10% (v/v) of
191 the final fermentation volume. Finally, fermentations were carried out in 250 mL
192 Erlenmeyer flasks containing 100 mL of MRS broth at 30 °C and 100 rpm during 49h.
193 Aliquots of 3 mL were collected each 2h and the pH measured by a pHmeter BASIC
194 20+ (CRISOL, Alella, Barcelona Spain). All fermentations were performed by triplicate
195 and standard deviations reported in the text.

196 **2.5.2. Preparation of cell-free supernatants (CFS) and neutralization**

197 CFS from the aliquots previously withdrawn were obtained by two methods: A) pH
198 correction to 3.0 - 3.5 with HCl 1 M in order to promote the desorption of bacteriocin
199 molecules adsorbed on the cell wall of the producer cell, followed by centrifugation (3421
200 g, 15 min), filter-sterilization using 0.22 µm pore-size membranes (Millipore), and heat
201 treatment of the supernatant at 90°C for 5 min; B) correction of the pH to 6.0 - 6.5 NaOH 5
202 M in order to eliminate the antimicrobial action of the acids produced during the
203 fermentation process, followed by centrifugation (3421 g, 15 min), filter-sterilization using

204 0.22 µm pore-size membranes (Millipore), and heat treatment of the supernatant at 90°C
205 for 5 min.

206 **2.5.3. Determination of bacteriocin-producer strains by agar well diffusion assay**

207 The methodology employed to check the production of bacteriocins against *Listeria*
208 *monocytogenes* CECT-934 or *Enterococcus faecium* 101, 104 and 711 was reported by
209 Rodríguez et al. (2013).

210 Bioindicator strains were reactivated overnight in 250 mL Erlenmeyer flasks placed in
211 an orbital shaker (Optic Ivymen System, Comecta S.A., distributed by Scharlab, Madrid,
212 Spain) containing 50 ml of MRS or BHI broth, at 30°C and 150 rpm. Grown strains were
213 diluted to reach a concentration of approximately 10^5 UFC/mL, and employed for the well
214 diffusion assay. 2 mL of the dilution was added to 20 mL of sterilized MRS or BHI broth
215 plus 1% (w/v) agar, poured into Petri dishes, and allowed to solidify at room temperature.
216 Wells (5 mm in diameter) were punched out of the solidified agar with a sterile metal
217 cylinder and filled with 50 µL of CFS. After incubation at 30°C during 24h, the inhibition
218 zones were examined for clearing around the wells. The inhibition halos were measured
219 using a digital caliper.

220 All experiments were performed by triplicate and standard deviations reported in the
221 text.

222 **2.6. Effect of growth temperature of selected strains**

223 The influence of the temperature was studied by fermentations conducted in MRS broth
224 at 15 and 30°C during 24h.

225 **2.7. Evaluation of hemicellulosic liquors as culture medium to produce bacteriocins**

226 Hemicellulosic liquors obtained by prehydrolysis 2 of BSG type 2 were assayed as
227 culture media to produce bacteriocins using the strains *L. lactis* Tw11 and *E. mundtii*
228 Tw492 at 30°C during 49h. Culture media were formulated without supplementation, or

229 after supplementation with only 1g L^{-1} of Tween 80 or with 1 g L^{-1} Tween 80 and the salts
230 of MRS broth (K_2HPO_4, 2 g L^{-1} diammonium hydrogen citrate, 5 g L^{-1} CH_3COONa, 0.2 g
231 L^{-1} $MgSO_4.7H_2O$, 0.05 g L^{-1} $MnSO_4.2H_2O$). Hemicellulosic liquors were filter-sterilized to
232 avoid modifications by heat.

233 In order to check the anitimicrobial effect of bacteriocins in exhausted hydrolyzates, the
234 two methodologies described in section 2.6.2. were combined. Therefore, CFS were
235 obtained by pH correction to 3.0 - 3.5 with HCl 1 M l, followed by centrifugation (3421 g,
236 15 min). The supernatant pH was corrected to 6.0 - 6.5 using NaOH 5 M. Finally, samples
237 were filter-sterilized using 0.22 µm pore-size membranes (Millipore) followed by heat
238 treatment of the supernatant at 90°C for 5 min.

239 The agar well assay was done the same way than the section 2.5.3, but different
240 concentrations of *L. monocytogenes* were used: 10^5 UFC/ml, 10^6 UFC/ml or 10^7 UFC/ml.
241 All experiments were performed by triplicate and standard deviations reported in the text.

242 **2.8. Monitoring of growth and pH on hemicellulosic liquors as medium**

243 Growth curves of *E. mundtii* Tw492 and *L. lactis* Tw11 on hemicellulosic liquors were
244 obtained by counting of viable cells (CFU/mL) using serial dilutions in saline solution
245 (0.85 % w/v). One hundred mL of each dilution was spread out over the surface of plates
246 containing MRS broth with 1.5% agar using Drigalski handle. The plates were incubated
247 for 24 to 48h, and only those containing from 20 to 200 colonies were considered.
248 Subsequently, the values of the counts were converted to decimal log. The progressive
249 acidification of culture medium was measured by a pHmeter BASIC 20+ (CRISOL, Alella,
250 Barcelona Spain) during the whole fermentation period.

251 **2.9. Analytical methods**

252 The liquid phase of samples was employed for analysis of glucose, xylose, arabinose,
253 furfural, acetic acid, formic acid, xylitol, glycerol, oxalic acid, citric acid, tartaric acid,

254 lactic acid and ethanol analysis by High Performance Liquid Cromatography (HPLC)
255 (Agilent, model 1200, Palo Alto, CA) using a refractive index detector with an Aminex
256 HPX-87H ion exclusion column (Bio Rad 300 × 7.8 mm, 9 µ particles) with a guard
257 column, eluted with 0.003 M sulfuric acid at a flow rate of 0.6 mL/min at 50 °C. Five µL
258 of conveniently diluted samples were injected. Concentrations were obtained using the
259 corresponding calibration curve ranging between 0 and 12 g L^{-1}. All these compounds were
260 obtained from (Panreac Química Sau, Barcelona, España, y Sigma–Aldrich, St. Louis, MO,
261 USA).

262 Filtered samples were also used to analyze phenolic compounds such as 5-
263 (Hydroxymethyl)furfural (HMF), 4-Hydroxybenzaldehyde, caffeic acid, syringic acid, *p*-
264 coumaric acid, ferulic acid, vanillin, 2-Methoxy-4-vinylphenol, vanillic acid, vanillyl
265 alcohol by high-performance liquid chromatography (HPLC Agilent model 1200, Palo
266 Alto, CA, USA) with UV-diode array detection equipped with a Zorbax SB-Aq column
267 (Agilent, Palo Alto, CA, USA). The elution program was carried out at 35°C and the
268 wavelength set at 276 nm. The mobile phase at flow rate of 1mL/min comprised a mixture
269 of two solvents: 2.5% formic acid aqueous solution (v/v) (A); and 100% methanol (B). The
270 following elution profile was used: solvent A started at 100% holding 35 min, then
271 decrease to 52% at 5 min, continued used only solvent B at 16 min, returned to 100% of A
272 at 4 min and the column was re-equilibrated for 5 min before the next injection. Standards
273 used for calibration curve (in the range 0 to 1 g L^{-1}) were provided by Sigma–Aldrich, St.
274 Louis, MO, USA.

275 Recovered sugar yield: Y_S (g of substance that can be obtained from 100 g of BSG dry
276 matter) and hydrolysis efficiency: η (%) were calculated using respectively the Eqns (1)
277 and (2) defined by Mussatto and Roberto (2005, 2006):

278 $$Y_S = (C \times V/M) \times 100 \qquad (1)$$

279 $$\eta = (Y_S / Y_{max}) \times 100 \quad (2)$$

280 where C is the concentration of the component in the liquid phase (g L^{-1}), M is the
281 amount of BSG (dry matter) employed in the experiment (g), V is the volume of liquid
282 solution employed (L) and Y_{max} is the maximum yield of recovered sugars that can be
283 attained (g per 100 g dry matter).

284 **2.10. Statistical analysis**

285 Mean values of characterization of BSGs, type of hydrolyses and quantitative acid
286 hydrolyses were submitted to analysis of variance (ANOVA) by the Statistica Software
287 13.0. They were compared using the Tukey's test at significance level $P < 0.05$, and
288 different letters were used to label values with statistically significant differences among
289 them.

290 **3. Results and Discussion**

291 **3.1. Characterization of BSG**

292 Two materials were screened for their characterization. **Table 2** shows the chemical
293 composition of the materials selected in this work. Additional information published in
294 literature is also compiled with comparative purposes. Variations in composition can be
295 attributable to the variety of the barley grains used, harvest time, malting and mashing
296 conditions, and the quality and type of adjuncts (non-malt sources of fermentable sugars)
297 employed (Robertson et al., 2010; Santos et al., 2003).

298 In our case, BSG samples were collected after cooking, showing moistures of 74.5% ±
299 0.3. This high moisture combined with the composition of BSG promotes the growth of
300 microorganisms, mainly molds and yeasts. Therefore, the raw material was dried to a
301 moisture content of 9.1% ± 0.010 for Type A and 6.6 ± 0.265 for Type B, finding
302 significant differences ($P < 0.05$) between them.

303 The ash content was similar for both types of BSG, but significant differences ($P <$
304 0.05) were found between samples. Type B showed a slightly higher content (3.4% ±
305 0.084) than Type A (3.1% ± 0.032). Statistical differences ($P < 0.05$) were also found in
306 Carbon and Nitrogen contents, with higher values (48.3% ± 0.165 and 5.1% ± 0.074
307 respectively) in BSG Type A.

308 **Table 2** shows the polysaccharides of both samples. The cellulose content is given as a
309 function of the glucan percentage, whereas the hemicellulose content was determined by
310 the amount of arabinoxylan (xylan and arabinan), since acetic acid was not quantified in
311 our samples. As shown in **Table 2**, there are slight differences in the percentages of
312 polysaccharides, but the statistical analysis for the mean difference (Tukey's test) indicated
313 that there were no significant differences between the samples ($P > 0.05$) regarding the
314 content of cellulose, xylan and lignin. The only significant difference was found in
315 arabinan and consequently in hemicelluloses. Therefore, based on the cellulose,
316 hemicellulose, and lignin content, both samples could be considered equivalent
317 lignocellulosic materials and therefore susceptible to being treated with the same type of
318 thermochemical treatment. In general, the polysaccharide content of the samples used in
319 the present study was lower than those published in other works (see **Table 2**), particularly
320 the hemicellulosic content, but most of these studies were carried out with BSG 100% malt
321 (without attachments), reason why some differences with the works of the literature can be
322 expected.

323 BSG also contains several minerals. This measurement is important because heavy
324 metals can be toxic or inhibitory of some biotechnological processes. In addition, the
325 excess or defect of other ions can give a reference about the supplements that should be
326 added to the medium (Stanbury et al., 2017). Silicon, phosphorus, calcium and magnesium
327 are the most abundant (Lynch et al., 2016). In our materials, no quantifiable values of Co,

328 Ni, Cd, and Al were obtained (see Table 3), being below the limit of determination of the
329 equipment used in both raw materials (Co < 0.1; Ni < 0.6; Cd < 0.05, and Al < 32 mg/Kg).
330 The concentration of the rest of the analyzed mineral elements is summarized in Table 3. It
331 is outstanding the high level of Ca (2700 mg/Kg ± 191.5 in Type A and 2340 mg/Kg ±
332 87.8 in Type B) and Mg (1677.0 mg/Kg ± 110.2 in Type A and 1581.0 mg/Kg ± 99.7 in
333 Type B). The amount of these minerals depends on the type of soil in which barley was
334 grown, and the cereals used as malt attachments. As it can be seen in Table 3, there are
335 significant differences in the amount of some minerals and this may be the cause of the
336 significant differences in ash content. Mussatto and Roberto (2006) also investigated the
337 minerals composition of BSG generated from a process using 100% malted barley,
338 obtaining in all cases (but the exception of K) higher amounts of minerals. In particular it
339 can be pointed out the amounts of Si (10740.0 mg/Kg) and P (5186.0 mg/Kg), which were
340 not quantified in our work.

341 Arabinoxylan, the main non-cellulose polysaccharide, is proposed to be attached to the
342 cellulose fibrils via hydrogen bonding (Lynch et al., 2016). According to Mussatto and
343 Roberto (2005), around 70% of BSG hemicellulosic content is represented by a xylan
344 backbone, and the remaining 30% by an arabinan structure. In our case, this ratio was
345 maintained in both materials: 66.5% xylan and 33.5% arabinan in the hemicellulosic
346 fraction of BSG type A, and 66.1% xylan and 33.9% arabinan in type B.

347 **3.2. Hydrolytic treatment**

348 Taking into account that the objective of this work was the use of the hemicellulosic
349 sugars, the hydrolytic treatment was assayed on bagasse type B due to the slightly higher
350 content of xylan (14. 2 % ± 1.076) and arabinan (7.3 % ± 0.201) in this material in
351 comparison with the material type A (11.7 % ± 1.296 and 5.9 % ± 0.820 respectively).
352 This hydrolytic treatment allows solubilizing the hemicellulosic fraction (xylan and

353 arabinan) into the corresponding monomers (xylose and arabinose respectively), obtaining
354 therefore hemicellulosic liquors susceptible to be used for the production of bacteriocins.
355 Two hydrolysis conditions were assayed as previously described. **Table 4** shows that
356 although liquors obtained under the milder conditions (prehydrolysis 1) showed slightly
357 higher concentrations of xylose (12.8 g L^{-1} ± 0.335) and arabinose (8.2 g L^{-1} ± 0.276), no
358 significant differences were observed between the amounts of pentoses obtained with both
359 treatments ($P > 0.05$). Therefore, two additional parameters were calculated: the recovered
360 sugars yield (Y_S) and the hydrolysis efficiency (η), and the information was also
361 incorporated in **Table 4**. Considering these parameters, the harsher conditions
362 (prehydrolysis 2) were selected as the best treatment since more sugars were recovered: 3.5
363 g glucose, 11.6 g xylose and 6.9 g arabinose per 100 g of BSG dry matter in treatment 2 vs
364 3.4 g glucose, 10.2 g xylose and 6.6 g arabinose per 100 g of BSG dry matter in treatment
365 1, which represents higher hydrolysis efficiencies: 14.3% glucose, 81.6% xylose and
366 94.70% arabinose in treatment 2 vs 14.08% glucose, 72.01% xylose and 90.00% arabinose
367 in treatment 1.
368 Furthermore, the hydrolysis efficiency of arabinan into arabinose (94.70%) was superior
369 to that of xylan into xylose (81.60%). Carvalheiro et al., (2004) suggested that this
370 behavior can be explained considering that arabinose exhibits a higher thermal sensitivity
371 than xylose, being therefore released first from the hemicellulose structure.
372 These results are similar to those achieved by Mussatto and Roberto (2006) that
373 achieved the best results of prehydrolysis (13.21 g L^{-1} xylose and 8.21 g L^{-1} arabinose)
374 using a liquid to solid ratio of 10 g/g and 120 mg H_2SO_4/g dry matter.
375 In addition, glucose and maltose were also released in lower amounts than xylose and
376 arabinose (see **Table 4**). Once again, higher amounts of glucose (4.3 g L^{-1} ± 0.137) and
377 maltose (2.1 g L^{-1} ± 0.077) were attained in prehydorlysis 1. Glucose could be originated

from the cellulose or hemicellulose fractions although Mussatto and Roberto (2005) speculated that is obtained mainly from hemicelluloses, because the glucose from cellulose is not usually hydrolyzed under the conditions commonly used for diluted acid hydrolysis. On the other hand, the maltose released could derive from non-solubilized fractions of malted barley.

Finally, Table 4 also reveals the presence of 1.0 g L^{-1} of acetic acid, a compound that appears in hydrolyzates when acetyl groups are attached. The acetyl groups are linked to the xylan backbones, and when these are depolymerized, the acetic acid is liberated to the reaction medium. Therefore, the content of acetyl groups in the solids decreases along the time, increasing the amount of acetic acid in the liquid medium. However, not all acetyl groups are released, and therefore, depending on the hydrolytic conditions some of them remain attached to the xylane backbone (Carvalheiro et al., 2004).

On the other hand, Table 4 also summarizes the composition of the untreated materials, quantified by quantitative acid hydrolysis of the solid residual fractions. In agreement with the results observed in the liquid fraction (higher amount of released compounds in prehydrolysis 1), lower percentages of untreated polymers (3.9% ± 0.456 glucan; 5.0% ± 0.641 xylan and 1.6% ± 0.217 arabinan) were observed using the first methodology.

3.3. Screening of Patagonian strains grown on MRS broth for bacteriocins production

A total of 17 strains belonging to the species *E. faecium, L. lactis, E. mundtii, P. pentosaceus, E. durans/hirae and E. faecalis* were evaluated in this work as bacteriocins producers in MRS broth. Fourteen of the microorganisms belong to the genus *Enterococcus*. Bacteriocins produced by this genus are known as enterocins, being their stability under extreme conditions and their activity during extended periods of time their most notable advantages (Ness et al., 2014; Schelegueda et al., 2015).

402 Two methodologies were assayed. In the first one, the pH of CFS was corrected to 3.0-
403 3.5 to allow the desorption of bacteriocin molecules adsorbed on cell walls, meanwhile in
404 the second process the pH was corrected to 6.0-6.5 in order to eliminate the antimicrobial
405 action of the acids produced during the fermentation process. Table 3 summarizes this
406 information. Additionally, negative control assays were conducted in parallel with aliquots
407 of non-inoculated MRS broth (with pH adjusted to 3.0 - 3.5 or 6.0 - 6.5) following the
408 same protocols. None of these aliquots exhibited any antimicrobial activity against the
409 bioindicator microorganisms.
410 The five microorganisms with an ewes' milk origin (*E. faecium* Etw6, Etw7, Etw15,
411 Etw20 and Etw22) did not show bacterial activity. Something similar happened with other
412 four strains: *L. lactis* Tw35, *P. pentosaceus* Tw224, *L. mesenteroide* Tw234 and *E.*
413 *durans/hirae* 463. The eleven remaining microorganisms showed antimicrobial activity
414 against the bioindicator microorganisms *Listeria monocytogenes* CECT-934 and the three
415 *Enterococcus faecium* assayed, although in general, the inhibitory halos were considerably
416 higher with *Listeria monocytogenes* CECT-934.
417 As expected, in most of the cases, the inhibitory halos were higher in the treatment A,
418 where the inhibitory action can also be ascribed to the acid produced through the
419 fermentation. Therefore, it was concluded that both treatments should be performed
420 sequentially in order to allow the liberation of adsorbed bacteriocin molecules at the time
421 that the influence of the produced acids is avoided.
422 Among the bacteriocins-producer strains, *E. mundtii* Tw492 provided the higher halos
423 of inhibition against *L. monocytogenes* CECT-934: 26.10 mm ± 0.283 in treatment A and
424 26.32 mm ± 0.233 in treatment B, no finding significant differences between them ($P >$
425 0.05). Consequently, these results prompted us to select this microorganism to be
426 fermented in the hemicellulosic liquors obtained during the prehydrolysis 2. Bennik et al.

427 (1998) reported for the first time the production of the bacteriocin mundticin by *E. mundtii*
428 and showed as this bacteriocin was able to inhibit some strains of *L. monocytogenes*,
429 *Clostridium botulinum* and some LAB. The ability to inhibit the growth of *Listeria* spp. is
430 common to most *Enterococcus* bacteriocins due to the close phylogenetic relationship of
431 enterococci and listeriae (Rivas et al., 2012). Other works have used this strain to describe
432 the characteristics of mundticin and particularly its potential use as a biocontrol agent in
433 foods, mainly due to its anti-listerial activity (Bigwood et al., 2012; Kawamoto et al., 2002;
434 Schelegueda et al., 2015; Settanni et al., 2014; Todorov and Dicks, 2009).
435 With comparative purposes, *L. lactis* Tw11 was also employed, considering that this
436 strain produces nisin, the bacteriocin most widely studied (Keren et al., 2004). Nisin can be
437 used as biopreservative in the food industry due to its antibacterial properties, which allows
438 stricter microbial controls of a variety of commercial food products (Khan and Oh, 2016).

439 **3.4. Influence of temperature on the production of bacteriocins using the Patagonian**
440 **strains *L. lactis* Tw11 and *E. mundtii* Tw492**

441 Several authors explained the influence of some abiotic parameters as temperature and
442 pH on the bacteriocin production (Balciunas et al., 2013; Parente and Ricciardi, 1999).
443 Todorov and Dicks (2009) reported that the production of several bacteriocins such as
444 lactocin A, enterocin 1146, lactocin S, amylovorin 1471 and nisin Z is correlated with
445 growth temperature. Therefore, the influence of growth temperature was evaluated against
446 *Listeria monocytogenes* CECT-934.
447 In our case, *L. lactis* Tw11 showed an increment with the reduction of temperature
448 from 16.76 mm ± 0.141 at 30°C to 18.99 mm ± 0.764 at 15°C. In agreement with these
449 results, Sequeiros et al. (2010) also observed the significant influence of the incubation
450 temperature when the strain *Lactococcus lactis* TW34 (a strain with similar origin) was
451 grown in MRS medium. Therefore, the highest antimicrobial production was found at

452 15°C/20°C in spite of the fact that these were not the optimal temperatures of growth. Such
453 activity decreased as the temperature increased, and therefore, at 25°C this activity was
454 reduced 4-fold.

455 Similarly, lacticin RM, a bacteriocin produced by *Lactococcus lactis* subsp. *lactis* EZ26
456 isolated from goat's milk, was optimally synthetized at low growth temperatures, with
457 maximal inhibition zones at 10 and 15°C. None activity was detected at 37°C, and only a
458 faint clear zone was obtained at 30°C, which was the optimal growth temperature (Keren et
459 al., 2004). Accordingly, although most of the characterized bacteriocins are produced at the
460 producer's optimal growth temperature, according to these authors, higher production at
461 suboptimal temperatures has been reported for some strains.

462 In contrast, *E. mundtii* Tw492 recorded the higher inhibition (30.31 mm ± 0.870) at
463 30°C. Meanwhile, a smaller activity was quantified at 15°C (23.87 mm ± 0.651). Todorov
464 and Dicks, (2009) using *Enterococcus mundtii* ST4SA also reported 8-fold higher levels of
465 peptide ST4SA at 30°C and 37°C than at 25°C, suggesting that growth temperature seems
466 to play an important role. Settanni et al., (2008) also observed that maximal production of
467 antilisterial compounds with *E. mundtii* was obtained in the optimal range of growth (30–
468 37°C).

469 **3.5. Use of BSG hemicellulic liquors to produce bacteriocins using the Patagonian**
470 **strains *L. lactis* Tw11 and *E. mundtii* Tw492**

471 The strains *L. lactis* Tw11 and *E. mundtii* Tw492 were grown in hemicellulosic
472 hydrolyzates obtained by Prehydrolysis 2 of BSG type 2. In spite of the best results
473 attained at lower temperatures in the case of *L. lactis*, in order to simplify the experimental
474 work, both microorganisms were grown at 30°C. Three strategies were assayed: a) absence
475 of supplementation; b) supplementation with 1 g L^{-1} Tween 80, and c) supplementation
476 with 1 g L^{-1} Tween 80 plus all the salts present on the MRS broth. Cell free supernatants

(CFS) were evaluated for their inhibitory activity towards *Listeria monocytogenes* CECT-934. Negative controls were again assayed without producing anti-listeria activity as it can be seen in **Figure 1a**.

L. lactis Tw11 grown in hemicellulosic hydrolyzates, but in the absence of supplementation, was unable to produce antibacterial substances against *L. monocytogenes* (see **Figure 1b** and **Figure 2a**). Numerous studies support the idea that growth phase and bacteriocin production are also correlated (Balciunas et al., 2013; Parente and Ricciardi, 1999). However, variations in bacteriocin production have been observed under different chemical compositions of media employed (Li et al., 2002; Settanni et al., 2008; Zendo et al., 2005) Conversely, **Figure 2b** shows the growth curve and the profile of bacteriocin production by *E. mundtii* Tw492. It can be observed the generation of bacteriocins in the mid-exponential phase of growth (3-5h), the increment of bacteriocins at the end of the growth phase (8h) and the maxima levels of antimicrobial activity during the stationary phase of growth (12h) remaining constant during the 24h evaluated.

On the other hand, **Figure 3** summarizes the profiles of sugars consumption and lactic acid generated with time when both microorganisms were grown in hemicellulosic hydrolyzates without supplementation. It can be observed that sugars were hardly consumed by *L. lactis* Tw11 (20.95% glucose, 18.96% xylose and 16.12% arabinose) generating only 0.29 g L^{-1} ± 0.084 of lactic acid with a global volumetric productivity (Q_{LA}) of 0.0122 g L^{-1} ·h and a product yield ($Y_{LA/GXA}$) of 0.075 g/g (see **Table 5**). Meanwhile, *E. mundtii* Tw492, in spite of the negligible consumption of xylose (8.84%) and arabinose (6.84%), 61.34% of the initial glucose was consumed, thus allowing the production of 2.5 g L^{-1} ± 0.092 lactic acid (Q_{LA} = 0.102 g L^{-1} ·h; $Y_{LA/GXA}$ = 0.61 g/g) and inhibition halos of 6.05 mm ± 0.424 using 10^5 UFC/mL of *L. monocytogenes* CECT-934. The scarce consumption of sugars during the exponential phase was also observed by

502 Charalampopoulos et al., (2002) during the growth of several probiotic strains
503 (*Lactobacillus fermentum, L. reuteri, L. acidophilus and L. plantarum*). They also
504 observed that during the stationary phase all strains except *L. acidophilus* continued to
505 consume sugars and accumulate organic acids

506 In order to overcome this scarce production of bacteriocins, additional experiments
507 were performed adding 1 g L^{-1} of the surfactant Tween 80 to the hemicellulosic
508 hydrolyzates. **Figure 4** shows the consumption of sugars and the production of
509 bacteriocins with time. It is outstanding the increment in glucose consumption (76% in
510 both microorganisms), and in a lesser extent for xylose and arabinose (see **Table 5**). The
511 concentration of lactic acid increased up to 3.0 g L^{-1} ± 0.106 using *L. lactis* Tw11 and
512 remained similar (2.5 g L^{-1} ± 0.010) with *E. mundtii* Tw492, meaning that the strong
513 increment observed in bacteriocin production can only be ascribed to the effect of
514 bacteriocins and not to lactic acid. The presence of 1 g L^{-1} Tween 80 enhanced 3.4-fold
515 times the amount of bacteriocins (20.70 mm ± 0.175) using the same concentration of 10^5
516 UFC/mL of *L. monocytogenes*, and even under a lower dilution, corresponding to 10^7
517 UFC/mL of *L. monocytogenes*, it was quantified an inhibition halo of 16.00 mm ± 0.095.
518 **Figure 1c** shows clearly the formation of halos by CFS against 10^5 UFC/mL of *L.*
519 *monocytogenes*.

520 Different theories tried to explain the influence of this tensioactive. For instance,
521 Todorov and Dicks (2009) observed that the inclusion of Tween 80 in the growth medium
522 increased peptide ST4SA production by more than 50%, probably because it could change
523 the surface tension of the producer cell, thereby increasing the rate of peptide released
524 from the cell's surface. Keren et al. (2004) also indicated that Tween 80 does not stimulate
525 growth or bacteriocin production, but reduces the adsorption of lacticin RM to producer
526 cells, leaving no significant residual bacteriocin adsorbed to the cells. However, other

527 authors observed that Tween 80 stimulate the production rather than their release (Huot
528 and Petitdemange, 1996; Moretro et al., 2000). For example, these last authors observed
529 that Tween-80 was essential for growth of *Lactobacillus sakei* CCUG and bacteriocin
530 production. Therefore, no growth was obtained without supplementation with Tween-80;
531 0·11% Tween-80 was required for optimal growth, meanwhile the highest sakacin P
532 activity was obtained with 0·3% Tween-80.

533 A final set of experiments was conducted adding the salts present in the MRS broth to
534 hemicellulosic hydrolyzates containing 1 g L^{-1} Tween 80. Conversely to the expected, all
535 sugars were worst consumed with both strains, ranging between 52.63-54.85% for glucose,
536 15.14-17.02% for xylose and 9.58-12.77% for arabinose (see **Figure 5** and **Table 5**).
537 Consequently, only 2.1 g L^{-1} ± 0.047 lactic acid (Q_{LA} = 0.051 g L^{-1} ·h; $Y_{LA/GXA}$ = 0.71 g/g)
538 and 1.9 g L^{-1} ± 0.010 of lactic acid (Q_{LA} = 0.039 g L^{-1} ·h; $Y_{LA/GXA}$ = 0.48 g/g) were obtained
539 with *L. lactis* Tw11 and *E. mundtii* Tw492 respectively. However, the production of
540 bacteriocins was similar independently of the addition of salts. Therefore, both
541 microorganisms exhibited the maximum antimicrobial activity against *L. monocytogenes* at
542 7h (see **Figure 5**) without eliciting any loss of antimicrobial activity through the whole
543 process (up to 49h). The diameter of the inhibition halos were 14.65 mm ± 0.050 and 20.04
544 mm ± 0.230 respectively with *L. lactis* Tw11 and *E. mundtii,* being similar to those
545 recorded in previous experiments without supplementation with salts. Different dilutions
546 also provided equal results, demonstrating that in our case, the addition of salts (K_2HPO_4,
547 diammonium hydrogen citrate, CH_3COONa, $MgSO_4.7H_2O$ and $MnSO_4.2H_2O$) did not
548 influence on the production of bacteriocins, probably due to hemicellulosic hydrolyzates
549 are rich media.

550 LAB are microorganisms with complex peptidic sources requirements. Accordingly,
551 commercial media for culturing LAB contains high concentrations of tryptone, peptone,

552 meat extract or yeast extract. Therefore, studies conducted by Leroy and De Vuyst (2001),
553 Li et al. (2002), Settanni et al. (2008) and Zendo et al. (2005) among others, revealed that
554 bacteriocin production in MRS broths decreased in the absence of nitrogen sources. For
555 instance, Todorov and Dicks (2009) pointed highest antimicrobial activity of peptide
556 ST4SA in MRS broth supplemented, among others, with nitrogen sources such as as yeast
557 extract, tryptone or a combination of both, meanwhile Aasen et al. (2000) showed that the
558 growth rate of *Lactobacillus sakei* CCUG 42687 was increased when media were
559 supplemented with growing concentrations of yeast extract, but also the maximum
560 concentration of sakacin P produced.

561 However, these requirements could be suppressed when using other sources of proteins.
562 In this way, Vázquez et al., (2006 and 2008) using peptones obtained from several waste
563 protein sources (in particular fish and cooked cephalopod residues) improved the
564 bacteriocins production (nisin and pediocin), regarding the use of bactopeptones or
565 commercial media specifically recommended for LAB growth. Aspmo et al. (2005) also
566 tested that fish hydrolyzates performed equally well or better than commercial
567 extracts/peptones.

568 As previously indicated and recorded in **Table 2**, BSG type B is a proteins rich source,
569 containing around 25.7 % ± 0.813. This fraction could be partially solubilized during the
570 hydrolytic treatment, which justifies the no need of supplementation with additional
571 nitrogen sources. In this way, Niemi et al., (2013) reported that proteins can be extracted
572 from BSG through an alkaline treatment, therefore, becoming a suitable medium to
573 produce bacteriocins without the need of additional supplementation.

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Conclusions

Brewery-spent grain is a rich byproduct with interesting skills in the field of bacteriocins and acid lactic production. In this work, it was employed as feedstock for the generation of hemicellulosic hydrolyzates, and further production of bacteriocins by Patagonian strains. Results showed as *Lactococcus lactis* Tw11 and *Enterococcus mundtii* Tw492 are able to produce antilisterial compounds from hemicellulosic hydrolyzates. In addition, the supplementation of medium with tween 80 showed a positive effect over the production and/or released of produced bacteriocins. In summary, BSG could be postulated as a suitable and cheap medium for the bacteriocins production.

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757 **Table 1.** Cleaning halos formation (mm) around wells charged with cell-free supernatant extracts obtained from Patagonian strains against
758 bioindicator strains *E. faecium* 101, 104 and 711 and *L. monocytogenes* CECT-934 using two different methodologies: A) pH correction to 3.0-
759 3.5 with HCl 1 M in order to promote desorption of bacteriocin, and B) correction of the pH to 6.0 - 6.5 NaOH 5 M in order to eliminate the
760 antimicrobial action of the acids produced.

		E. faecium 101		*E. faecium* 104		*E. faecium* 711		*L. monocytogenes* CECT-934	
Denomination	**Origen**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
E. faecium Etw6	Ewes' milk	-	-	-	-	-	-	-	-
E. faecium Etw7	Ewes' milk	-	-	-	-	-	-	-	-
E. faecium Etw15	Ewes' milk	-	-	-	-	-	-	-	-
E. faecium Etw20	Ewes' milk cheese	-	-	-	-	-	-	-	-
E. faecium Etw22	Ewes' milk cheese	-	-	-	-	-	-	-	-
L. lactis Tw11	*Odontesthes platensis* (Berg)	16.72 ± 0.042^{a}	15.46 ± 0.064^{b}	16.36 ± 0.205^{a}	15.40 ± 0.049^{b}	16.63 ± 0.134^{a}	15.20 ± 0.212^{b}	16.76 ± 0.035^{a}	15.75 ± 0.042^{b}
L. lactis Tw35	*Odontesthes platensis* (Berg)	-	-	-	-	-	-	-	-
E. mundtii Tw56	*Odontesthes platensis* (Berg)	15.23 ± 0.042^{a}	14.66 ± 0.014^{b}	16.32 ± 0.028^{a}	15.65 ± 0.064^{b}	16.61 ± 0.042^{a}	15.23 ± 0.092^{b}	17.00 ± 0.035^{a}	16.48 ± 0.495^{a}
P. pentosaceus Tw224	*Odontesthes platensis* (Berg)	-	-	-	-	-	-	-	-
E. mundtii 278	*Holothuroidea*	14.70 ± 0.212^{a}	15.40 ± 0.071^{b}	15.01 ± 0.389^{a}	15.73 ± 0.346^{a}	15.20 ± 0.113^{a}	16.11 ± 0.156^{b}	16.55 ± 0.085^{a}	16.74 ± 0.000^{a}
E. faecium 452	*Mytilus edulis*	14.02 ± 0.198^{a}	14.38 ± 0.226^{a}	14.20 ± 0.064^{a}	14.60 ± 0.219^{a}	13.81 ± 0.184^{a}	14.18 ± 0.488^{a}	15.72 ± 0.078^{a}	15.87 ± 0.064^{a}
E. faecium 459	*Mytilus edulis*	12.32 ± 0.007^{a}	10.62 ± 0.255^{b}	13.09 ± 0.120^{a}	10.70 ± 0.184^{b}	12.48 ± 0.064^{a}	10.43 ± 0.106^{b}	14.89 ± 0.064^{a}	12.67 ± 0.042^{b}
E. durans/hirae 463	*Mytilus edulis*	-	-	-	-	-	-	-	-
E. faecium 465	*Mytilus edulis*	16.10 ± 0.021^{a}	16.29 ± 0.325^{a}	$15.580.134^{a}$	14.92 ± 0.113^{b}	15.33 ± 0.021^{a}	15.19 ± 0.247^{a}	25.43 ± 0.028^{a}	23.89 ± 0.396^{b}
E. faecium 468	*Mytilus edulis*	14.71 ± 0.375^{a}	14.39 ± 0.552^{a}	13.68 ± 0.177^{a}	13.61 ± 0.156^{a}	13.72 ± 0.014^{a}	13.86 ± 0.113^{a}	24.70 ± 0.184^{a}	24.27 ± 0.361^{a}
E. faecalis 471	*Mytilus edulis*	16.65 ± 0.325^{a}	17.34 ± 0.205^{a}	16.14 ± 0.141^{a}	16.39 ± 0.453^{a}	16.10 ± 0.106^{a}	15.73 ± 0.276^{a}	26.00 ± 0.035^{a}	22.46 ± 0.361^{b}
E. mundtii Tw492	*Mimus saturninus*	19.06 ± 0.453^{a}	19.14 ± 0.368^{a}	18.24 ± 0.177^{a}	18.07 ± 0.262^{a}	$18.060.057^{a}$	17.25 ± 0.226^{b}	26.10 ± 0.283^{a}	26.33 ± 0.233^{a}

761 Results are expressed as diameters of the inhibition zone and standard deviations in mm (filter disks had a diameter of 5 mm)
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765 **Table 2**. Chemical composition (in %) of BSG samples.

Component	This work (Type A)	This work (Type B)	Adeniran et al., (2010)	Kanauchi et al., (2001)	Khidzir et al., (2010)	Meneses et al. (2013)	Mussatto and Roberto, (2005)	Mussatto and Roberto, (2006)	Öztürk et al. (2002)			Russ et al., (2005)
									Fine	Medium	Coarse	
Humidity	9.1±0.010 [a]	6.6±0.265 [b]	6.4±0.2						7.3	7.0	6.9	
Ashes	3.1±0.032 [a]	3.4±0.050 [b]	7.9±0.1	2.4	2.3±0.8	4.18±0.03	4.6	4.6	2.54	3.15	3.69	4.0-4.5
Extractives		12.7±0.748				10.73±0.32	5.8	5.8				
C	48.3±0.165 [a]	47.2±0.341 [b]			35.6±0.3							
N	5.1±0.074 [a]	4.1±0.130 [b]			1.0±0.05							
Protein	31.1±0.461 [a]	25.7±0.813 [b]	2.4±0.2	24	6.4±0.3	24.69±1.04	15.3	15.3	36.3	21.3	13.0	19-23
Carbohydrates			79.9±0.6									
Cellulose	24.6±6.270 [a]	24.4±0.998 [a]		25.4		21.73±1.36	16.8	16.8				23-25
Hemicellulose	17.6±1.577 [a]	21.5±1.272 [b]		21.8		19.27±1.18	28.4	28.4	19.2	19.6	19.6	30-35
Xylan	11.7±1.296 [a]	14.2±1.076 [a]				13.63±0.82		19.9				
Arabinan	5.9±0.820 [a]	7.3±0.201 [b]				5.64±0.35		8.5				
Acetyl groups							1.4	1.4				
Lignin	13.1±2.000* [a]	13.4±0.544* [a]		11.9		19.40±0.34	27.8	27.8**	14.6	14.9	15.2	7-8
NDFC									49.9	51.1	52.4	
ADF					23.3				30.7	31.5	32.8	
TDF									60.3	63.6	71.2	
Crude Fibre			3.3±0.1									
Lipid				10.6	2.5±0.1							

766 Protein: N x 6.25; NDFC: Neutral Detergent Fibre Content; ADF: Acid Detergent Fibre; TDF: Total Dietary Fibre.

767 *Klason lignin

768 **23.8% Klason lignin and 4.9% acid-soluble lignin

769 Different letters (in BSG type A and B) mean statistically significant differences among values in the same row ($P < 0.05$).

770

771 **Table 3**. Mineral composition (in mg/Kg) of BSG samples.
772

Mineral	This work (Type A)	This work (Type B)	Meneses et al. (2013)	Mussatto and Roberto (2006)
Al	<32	<32	81.20±2.20	
B			3.20±0.80	
Ba			8.62±0.00	13.6
Ca	2700±191.5[a]	2340±87.8[b]	3600±0.00	3515.0
Cd	<0.05	<0.05	<0.15	
Co	<0.1	<0.1	17.77±0.22	
Cr	0.83±0.153[a]	0.77±0.058[a]	<0.54	5.9
Cu	11.3±1.301[a]	9.8±0.058[a]	11.40±0.50	18.0
Fe	118.0±9.165[a]	128.0±7.937[a]	154.90±0.60	193.4
Ga			<1.47	
I			11.00±0.72	
K	591.0±55.055[a]	1152.3±20.404[b]	600±0.00	258.1
Mg	1677.0±110.2[a]	1581.0±99.7[a]	1900±0.00	1958.0
Mo			1.35±0.34	
Mn	36.1±1.850[a]	28.2±1.222[b]	40.90±0.90	51.4
Na			137.10±6.10	309.3
Ni	<0.6	<0.6	<0.54	
P			6000±0.00	5186.0
Pb	0.03±0.006[a]	0.05±0.006[b]	<1.60	
S			2900±0.00	1980.0
Se			<1.60	
Si				10740.0
Sn			<1.30	
Sr			10.36±0.00	12.7
Zn	73.6±4.366[a]	61.9±1.234[b]	82.10±0.60	178.0

773

774 **Table 4**. Hydrolytic treatment of BSG type B: a) compounds released (in g L^{-1}) during the
775 prehydrolysis carried out under two different conditions and b) quantitative acid hydrolysis
776 of the residual solid material. Prehydrolysis 1: liquid/solid ratio of 8 g/g and 17 min at
777 120ºC and prehydrolysis 2: liquid/solid ratio of 10 g/g; 27 min at 120ºC.
778

	Prehydrolysis I			Prehydrolysis II		
a) Composition of hydrolyzates						
	Concentration (g L^{-1})	***Ys*** **(g/100 g BSG)**	***η*** **(%)**	**Concentration (g L^{-1})**	***Ys*** **(g/100 g BSG)**	***η*** **(%)**
Maltose	2.1± 0.077 [a]	1.7		1. 3 ± 0.116 [b]	1.3	
Glucose	4.3 ± 0.137 [a]	3.4	14.08	3.5 ± 0.197 [a]	3.5	14.32
Xylose	12.8 ± 0.335 [a]	10.2	72.01	11.6 ± 0.115 [a]	11.6	81.60
Arabinose	8.2 ± 0.276 [a]	6.6	90.00	6.9 ± 0.381 [a]	6.9	94.70
Glycerol	0.41 ± 0.044 [a]	0.328		0.39 ± 0.016 [a]	0.39	
Acetic acid	1.0 ± 0.003 [a]	0.80		1.0 ± 0.020 [a]	1.0	
b) Quantitative acid hydrolysis of the residual solid						
	Percentage (%)			**Percentage (%)**		
Glucan	3.9 ±0.456 [a]			5.2 ± 0.039 [a]		
Xylan	5.0 ±0.641 [a]			6.5 ± 0.082 [a]		
Arabinan	1.6 ±0.217 [a]			1.9 ± 0.029 [a]		
Klason lignin	56.1 ±0.168 [a]			54. 6 ± 0.184 [b]		

779 *Ys*: g of substance that can be obtained from 100 g of BSG dry matter; *η* (%): hydrolysis efficiency.
780 Different letters (in BSG type A and B) mean statistically significant differences among values in the same
781 row ($P < 0.05$).
782

783 **Table 5.** Data summarizing the consumption of sugars and the production of lactic acid in hemicellulosic hydrolyzates in absence or presence of
784 supplements using the strains *L. lactis* TW11 and *E. mundtii* 492. Bacteriocins were measured against *Listeria monocytogenes* CECT-934.
785

Strain	Supplementation			Glucose			Xylose			Arabinose			Lactic acid			Bacteriocin	
	Tween	Salts	T	$Glucose_{t=0}$	$Glucose_t$	Cons.	$Xylose_{t=0}$	$Xylose_t$	Cons.	$Arab_{t=0}$	$Arab_t$	Cons.	LA_t	Q_{LA}	$Y_{LA/GXA}$	Conc.	Halo*
			(h)	($g\ L^{-1}$)	($g\ L^{-1}$)	(%)	($g\ L^{-1}$)	($g\ L^{-1}$)	(%)	($g\ L^{-1}$)	($g\ L^{-1}$)	(%)	($g\ L^{-1}$)	($g\ L^{-1}\cdot h$)	(g/g)		(mm)
Tw11	-	-	24	3.5±0.190	2.8±0.490	20.95	10.7±0.303	8.7±1.639	18.96	6.9±0.349	5.8±1.141	16.12	0.29±0.084	0.0122	0.075	10^7	0
Tw492	-	-	24	4.2±0.100	1.6±0.148	61.34	10.7±0.048	9.7±0.293	8.84	7.8±0.192	7.3±0.060	6.84	2.5±0.092	0.102	0.61	10^6	6.05±0.424
Tw11	✓	-	49	3.4±0.009	0.79±0.026	76.51	10.0±0.226	7.5±0.256	25.17	7.8±0.143	6.3±0.164	18.17	3.0±0.106	0.062	0.47	10^5	13.57±0.020
Tw492	✓	-	49	3.4±0.009	0.80±0.005	76.26	10.0±0.026	6.8±0.024	31.83	7.8±0.143	5.6±0.007	27.71	2.5±0.010	0.042	0.26	10^7	16.00±0.095
																10^6	20.70±0.175
																10^5	21.89±0.055
Tw11	✓✓	✓✓	49	3.4±0.080	1.6±0.017	52.63	7.5±0.147	6.4±0.012	15.14	6.6±0.161	6.0±0.008	9.58	2.1±0.047	0.051	0.71	10^5	14.65±0.050
Tw492	✓✓	✓✓	49	3.4±0.080	1.5±0.012	54.85	7.5±0.147	6.3±0.046	17.02	6.6±0.161	5.8±0.003	12.77	1.9±0.010	0.039	0.48	10^7	16.83±0.090
																10^6	20.04±0.230
																10^5	20.70±0.190

786 *Results are expressed as diameters of the inhibition zone and standard deviations in mm (filter disks had a diameter of 5 mm)

787 t: time where bacteriocins were evaluated; $Glucose/Xyloser/Arabinose_{t=0}$: glucose, xylose or arabinose at the beginning of the process; $Glucose/Xylose/Arabinose_t$: residual amount of
788 glucose, xylose or arabinose at time t; Cons. Percentage of glucose, xylose or arabinose consumed; LA: concentration of lactic acid at time t; Q_{LA}: global volumetric productivity of LA,
789 defined as $(LA_t - LA_{t=0})/t$; $Y_{LA/GXA}$: LA yield, calculated as $(LA_t - LA_{t=0})/((Glucose_{t=0} - Glucose_t) + (Xylose_{=0} - Xylose_t) + (Arabinose_{t=0} - Arabinose_t))$.

790 **Figure legends**

791 **Figure 1.** Halo formation by CFS against 10^5 UFC/mL of *L. monocytogenes*: A) Negative
792 control assays with aliquots of non-inoculated hemicellulosic liquor; B) Halo formation by
793 *L. lactis* Tw11 grown in hemicellulosic liquor without supplementation; and C) Halo
794 formation by *E. mundtii* Tw492 grown in hemicellulosic liquor supplemented with Tween
795 80.

796 **Figure 2.** Production of bacteriocins (inhibition halos against *Listeria monocytogenes*
797 CECT-934) and growth kinetic of a) *L. lactis* Tw11 and b) *E. mundtii* Tw492. Experiments
798 were performed at 30ºC in hemicellulosic hydrolyzates without supplementation.

799 **Figure 3.** Profile of sugars consumption and lactic acid and bacteriocin production by a) *L.*
800 *lactis* Tw11 and b) *E. mundtii* Tw492 grown in hemicellulosic hydrolyzates obtained by
801 Prehydrolysis 2 of BSG type 2 without supplementation.

802 **Figure 4.** Profile of sugars consumption and lactic acid and bacteriocin production by a) *L.*
803 *lactis* Tw11 and b) *E. mundtii* Tw492 grown in hemicellulosic hydrolyzates obtained by
804 Prehydrolysis 2 of BSG type 2 supplemented with 1 g L^{-1} Tween 80. Bar with horizontal
805 lines correspond to consumption of glucose, white bar to consumption of xylose, dotted bar
806 to arabinose consumption and grey bar correspond to production of lactic acid. The lines
807 represented the halo produced (mm) against: Straight line (10^5 UFC/mL of *Listeria*
808 *monocytogenes* CECT-934, Striped line (10^6 UFC/ml) and Dotted line (10^7 UFC/ml).

809 **Figure 5.** Profile of sugars consumption and lactic acid and bacteriocin production by a) *L.*
810 *lactis* Tw11 and b) *E. mundtii* Tw492 grown in hemicellulosic hydrolyzates obtained by
811 Prehydrolysis 2 of BSG type 2 supplemented with 1 g L^{-1} Tween 80 plus all the salts
812 present on the MRS broth. Bar with horizontal lines correspond to consumption of glucose,
813 white bar to consumption of xylose, dotted bar to arabinose consumption and grey bar
814 correspond to production of lactic acid. The lines represented the halo produced (mm)
815 against: Straight line (10^5 UFC/mL of *Listeria monocytogenes* CECT-934, Striped line (10^6
816 UFC/ml) and Dotted line (10^7 UFC/ml).

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822 **Figure 1**

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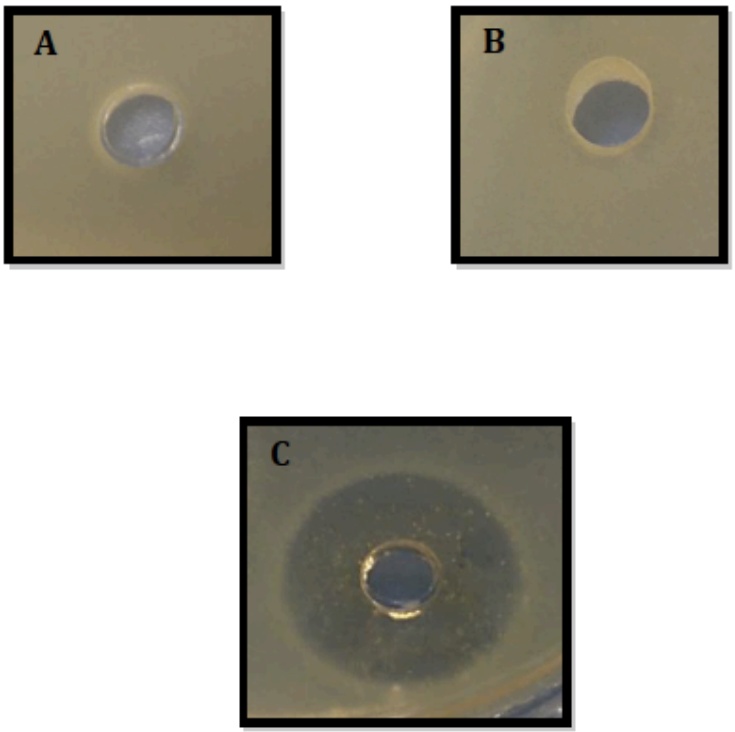

840 **Figure 2.A**
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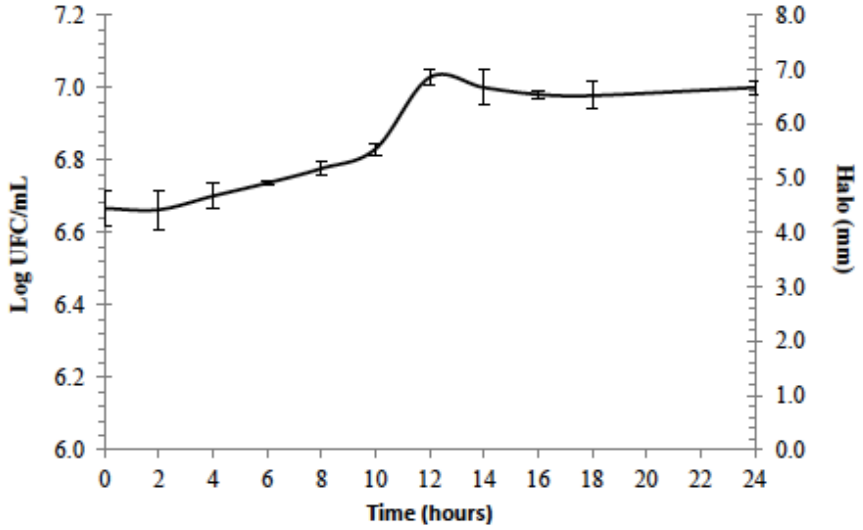

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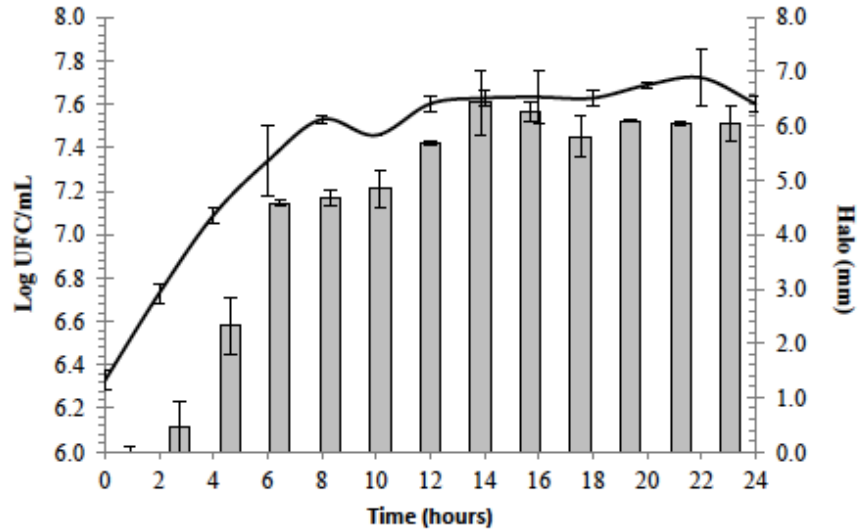
845 **Figure 2.B**



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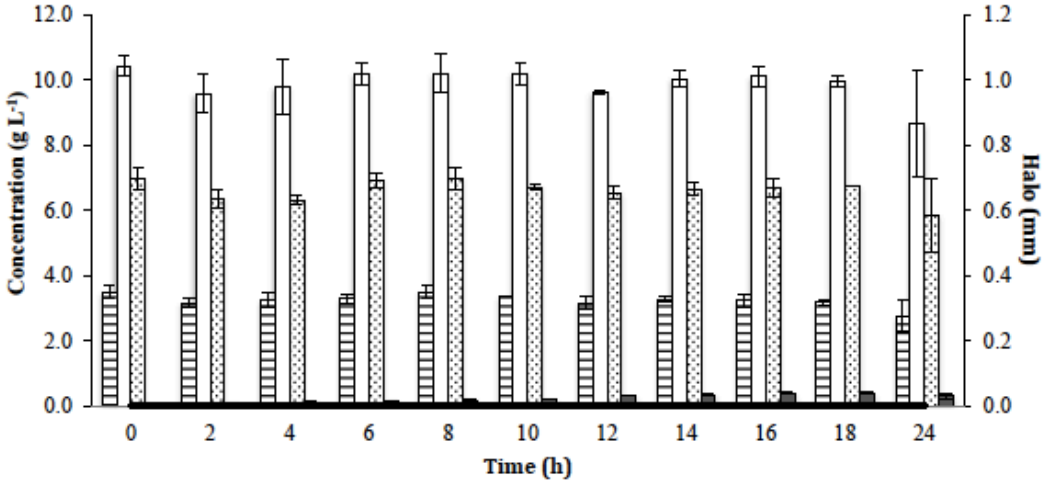
847 **Figure 3.A**



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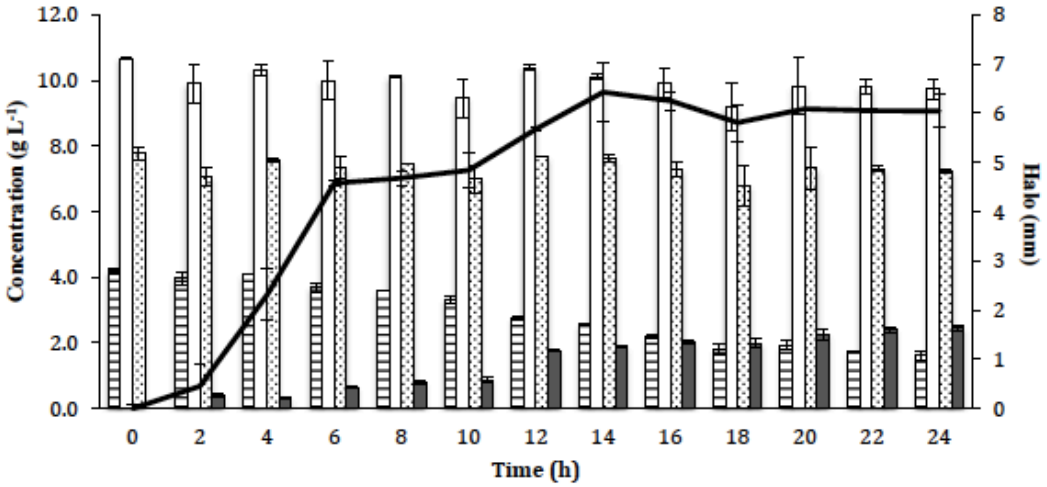
852 **Figure 3.B**



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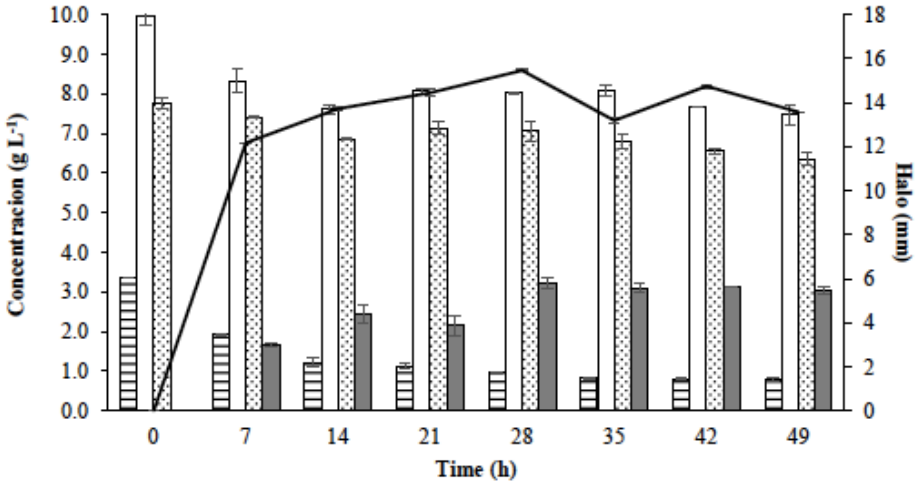
854 **Figure 4A**



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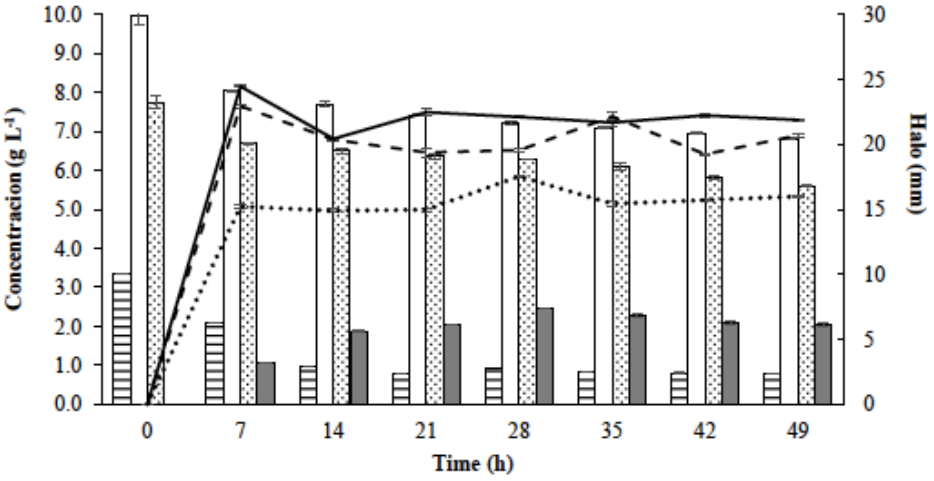
862 **Figure 4.B**



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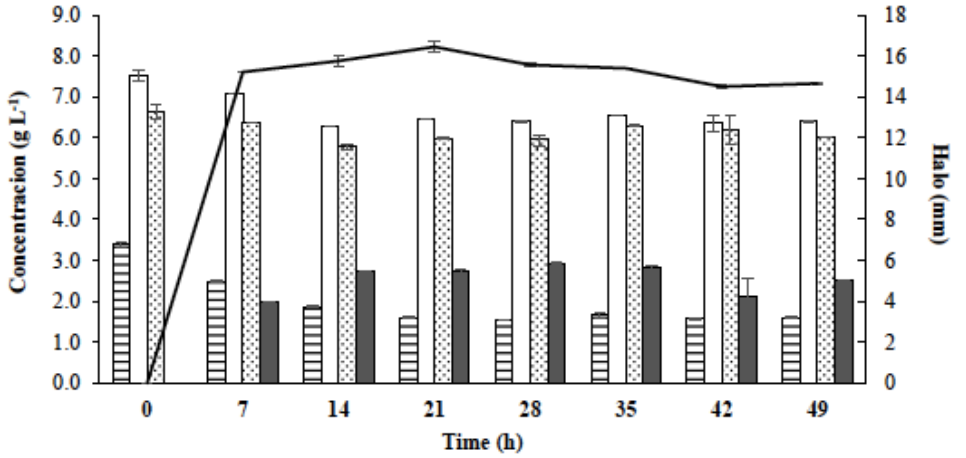
865 **Figure 5.A**



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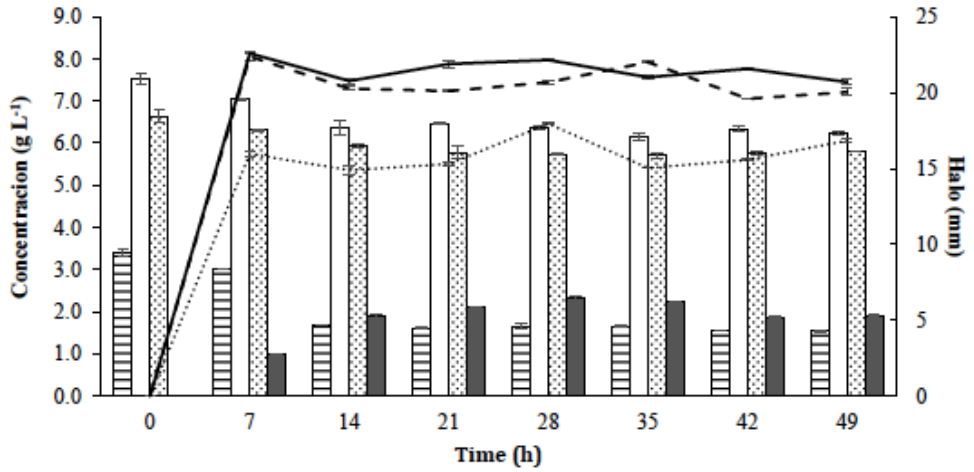
873 **Figure 5.B**



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APPENDIX B – Published article in collaboration: Optimisation of cheese whey enzymatic hydrolis and further continuous of antimicrobial extracts by *Lactobacillus plantarum* CECT-221.

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Optimisation of cheese whey enzymatic hydrolysis and further continuous production of antimicrobial extracts by *Lactobacillus plantarum* CECT-221

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The enzymatic hydrolysis of cheese whey was optimised using the enzymes iZyme, Alcalase or Flavourzyme under different conditions. Hydrolysates supplemented with commercial nutrients were evaluated as fermentation broths to produce DL-3-Phenyllactic acid (PLA) from phenylalanine (Phe) by *Lactobacillus plantarum* CECT-221. Optimised hydrolysates were obtained using Flavourzyme at 50 °C and 100 rpm during 12 h, and assayed in 250 ml Erlenemyer flasks using different proportions of vinasses as economic nutrient. The process was then scaled up using a 2 litres Bioreactor working under the continuous modality. Under the intermediate dilution rate of 0·0207 h^{-1} 0·81 ± 0·026 mM of PLA and 38·8 ± 3·253 g/l of lactic acid were produced. A final evaluation revealed that lactic acid, and bacteriocins exerted the highest inhibitory effect among the extracted components of cell-free supernatants.

Keywords: Cheese whey, vinasses, antimicrobial extracts, *Lactobacillus plantarum*, phenyllactic acid, bacteriocins.

Biopreservation, the use of microorganisms and/or their metabolites to prevent spoilage and to extend the shelf life of foods, has growing interest due to the continuous request for food-grade chemical antifungal agents (Cortés-Zavaleta et al. 2014). The use of naturally occurring antimicrobials in foods retains the nutritive value of food without producing side effects (Kumar et al. 2013). In this context, lactic acid bacteria (LAB) produce a variety of antifungal substances such as organic acids, proteinaceous compounds and various low-molecular mass substances, including DL-3-Phenyllactic acid (PLA) (Yang & Chang, 2010), an organic acid by-product of phenylalanine metabolism that has been produced by several microorganisms, particularly using some strains of LAB (Valerio et al. 2004; Li et al. 2007; Prema et al. 2008; Mu et al. 2009; Zheng et al. 2011; Rodríguez et al. 2012; Rodríguez-Pazo et al. 2013). Due to its broad inhibitory activity against a variety of food-borne microorganisms, PLA has interesting potential for practical application as an antimicrobial agent in the food industry (Mu et al. 2009) providing new perspectives for the possibility of using this natural antimicrobial compound to control fungal contaminants and extend the shelf life of food and/or feedstuffs (Lavermicocca et al. 2003).

In spite of their wide application in the food industry, LAB are catalogued as fastidious-growing microorganisms with numerous requirements for growth including amino acids, peptides, vitamins, and nucleic acids (Brinques et al. 2010). Thus, although industry and consumers for food-related applications prefer products obtained by biotechnological procedures, fermentation technologies must be cost competitive with chemical synthesis to carry out the biotechnological process at an industrial scale (Bustos et al. 2004). Therefore, alternative low-cost media should be developed to efficiently compete with the costly synthetic media for large-scale commercial applications (Brinques et al. 2010). In this sense, cheese whey and vinification lees, two by-products of the dairy and wine industries, respectively, could be assayed to formulate economic fermentative media.

Considering that cheese whey is a carbohydrate reservoir of lactose and also contains essential nutrients, it can be

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employed as a cheap carbohydrate source for the biotechnological production of biomass or high-added extracellular products (Brinques et al. 2010; Panesar et al. 2010; Rodríguez-Pazo et al. 2013). On the other hand, vinasses, the main liquid wastes from the distillation process, are also alternative sources of financially competitive nutrients already efficiently employed for the economical production of food additives (Bustos et al. 2004; Salgado et al. 2009).

Among LAB, *Lactobacillus plantarum*, a heterofermentative metabolism with high acid tolerance and generally regarded as safe organism (GRAS), is one of the most widespread species used in food technologies. In this work, the production of PLA by *L. plantarum* was evaluated using cheese whey as an inexpensive source of carbon (lactose) and phenylalanine in batch cultures, after optimisation of the cheese whey enzymatic hydrolysis using three enzymes: iZyme, Flavourzyme and Alcalase. Vinasess were assayed as alternative economic nutrients to provide nitrogen compounds, mineral salts, and essential nutrients for microbial growth in batch or continuous fermentations. Finally, the antimicrobial activity of the cell-free supernatants was assayed against *Carnobacterium piscicola*.

Materials and methods

Materials

Cheese whey, kindly provided by the cheese plant Ruta Xacobea S.L. in Brea (A Coruña, Spain), and wine lees, kindly supplied by the winery Adegas San Roque (Beade, Ourense, Spain) were stored at 4 °C until use.

Cheese whey enzymatic hydrolysis

Flavourzyme (EC 3.4.11.1) with endo- and exoprotease activities, and Alcalase (EC 3.4.21.62) and iZyme BA (EC 3.4.21.1) with endoprotease activities, kindly provided by Novozymes (Copenhagen, Denmark), were used to carry out the enzymatic hydrolysis of the protein fraction of cheese whey in order to improve the substrate (phenylalanine) concentration and/or protein co-substrate that could stimulate the PLA production. The enzymatic hydrolysis was carried out for 1 or 12 h at 50 °C in a thermostatic bath, using the necessary amount (V) of enzyme to hydrolyse 10 g/l of protein. Three enzymes were assayed according to the following activities: flavourzyme with activity equivalent to 1000 Leucine aminopeptidase units (LAPU)/g; alcalase with activity equivalent to 2·4 Anson units (AU)/g, and iZyme BA with activity equivalent to 0·15 Anson units (AU)/g. Additional experiments were also conducted using 2 V. In all cases, cheese whey was initially adjusted to pH 7 with 5 N NaOH. Table 1 summarises all the experiments conducted.

Strains, growth conditions and inoculum preparation

Lactobacillus plantarum CECT-221, obtained from the Spanish Collection of Type Cultures (Valencia, Spain), was employed to assess its ability to produce different antimicrobial metabolites, meanwhile *Carnobacterium piscicola* CECT 4020 was employed as the target organism to evaluate the bacteriocin activity of commercial acids and cell-free supernatants (CFS) obtained after fermentation processes. Seeds of *L. plantarum* or *C. piscicola* were activated in 250 ml Erlenmeyer flasks using 100 ml of the Man-Rogosa-Sharpe (MRS) medium containing 10 g/l peptone, 8 g/l beef extract, 4 g/l yeast extract, 20 g/l D(+)-glucose, 2 g/l K_2HPO_4, 2 g/l diammonium hydrogen citrate, 5 g/l CH_3COONa, 0·2 g/l $MgSO_4{\cdot}7H_2O$, 0·05 g/l $MnSO_4{\cdot}2H_2O$ and 1 g/l Tween-80 at 31·5 °C and 100 rpm in orbital shakers according to the conditions reported by Rodríguez-Pazo et al. (2013). Aliquots of culture (0·5 ml) from the late log/stationary phase were dispensed into cryovials and maintained at −80 °C using 15% (v/v) glycerol as cryoprotectant.

Inocula to carry out fermentations were prepared by transferring one stock cryovial into 250 ml Erlenmeyer flasks containing 100 ml of activation medium. Seed activation cultures were grown for 12 h at 31·5 °C and 100 rpm in orbital shakers. At least two generations of activation cultures were required before inoculation. The cells from this culture were finally recovered by centrifugation at 2755 ***g*** for 15 min at 10 °C and used to inoculate fermentations with 5% (v/v) of the final fermentation volume. pH was initially corrected to 6·2 with 5 N NaOH and sterilised at 101 °C for 60 min in autoclave.

Fermentation of cheese whey

Raw cheese whey and all the hydrolysates obtained after enzymatic hydrolysis were assayed as fermentation media for the formation of PLA by *L. plantarum*. Cheese whey hydrolysates (100 ml) were supplemented with the nutrients of Brinques et al. (2010) containing 15 g/l peptone, 5 g/l yeast extract, 0·2 g/l $MgSO_4{\cdot}7H_2O$ and 0·04 g/l $MnSO_4{\cdot}2H_2O$, or remained without nutrients addition, to evaluate the optimal hydrolysis conditions.

Optimised hydrolysates were supplemented with vinasses. Before use, vinasses were centrifuged and the liquid fraction neutralised with NaOH to pH 7. The liquid fraction was frozen, and subsequently freeze-dried. The solid fraction was keep at refrigerator. Different volumes of freeze-dried vinasses (25, 50, 75, 100 ml) or 50 ml plus 20 g/l of solid fraction were resuspended in 100 ml final volume of previously hydrolysed cheese whey, to assess the possibility of use vinasses as only and economic nitrogen and mineral salts source for fermentation process.

All fermentations were carried out placing 250 ml Erlenmeyer flasks (containing 100 ml of culture medium) in orbital shakers at 31 °C and 100 rpm. Samples were withdrawn at different intervals to monitor fermentation for up to 80 h. Initially, 20 g/l of calcium carbonate was added to neutralise the acids produced.

Additional experiments were performed in continuous fermentation using a 2 l Braun Biostat fermenter at 31·5 °C

Table 1. Influence of nutrients addition and conditions of cheese whey enzymatic hydrolysis on fermentation parameters

Run	Enzyme	Time EH (h)	*V*	Nut.	Time of ferment. (h)	$Phe_{t=0}$ (mg/l)	Phe_{final} (mg/l)	PLA (mM)	Q_{PLA} (mg/l·h)	$Y_{PLA/Phe}$ (g/g)
(a) F1	–	–	–	–	78	$4{\cdot}3 \pm 0{\cdot}283^{a}$	$104{\cdot}3 \pm 6{\cdot}505^{c}$	$0{\cdot}017 \pm 0{\cdot}006^{a}$	$0{\cdot}036 \pm 0{\cdot}012^{a}$	–
(a) F2	–	–	–	+	71	$152{\cdot}9 \pm 6{\cdot}364^{bc}$	$21{\cdot}4 \pm 5{\cdot}798^{a}$	$0{\cdot}93 \pm 0{\cdot}085^{fg}$	$2{\cdot}188 \pm 0{\cdot}199^{def}$	$1{\cdot}18 \pm 0{\cdot}102^{ab}$
(b) F3	Alc.	1	V	–	54	$11{\cdot}2 \pm 0{\cdot}283^{a}$	$25{\cdot}4 \pm 8{\cdot}485^{a}$	$0{\cdot}035 \pm 0{\cdot}008^{ab}$	$0{\cdot}108 \pm 0{\cdot}026^{a}$	–
(b) F4	Flav.	1	V	–	78	$102{\cdot}6 \pm 4{\cdot}243^{b}$	$7{\cdot}7 \pm 1{\cdot}980^{a}$	$0{\cdot}57 \pm 0{\cdot}099^{cde}$	$1{\cdot}215 \pm 0{\cdot}211^{bcd}$	$0{\cdot}99 \pm 0{\cdot}108^{a}$
(b) F5	Izy.	1	V	–	78	$5{\cdot}9 \pm 0{\cdot}566^{a}$	$6{\cdot}9 \pm 1{\cdot}556^{a}$	$0{\cdot}093 \pm 0{\cdot}008^{ab}$	$0{\cdot}198 \pm 0{\cdot}018^{ab}$	–
(b) F6	Alc.	1	V	+	46	$181{\cdot}8 \pm 4{\cdot}808^{c}$	$109{\cdot}4 \pm 16{\cdot}829^{c}$	$0{\cdot}77 \pm 0{\cdot}085^{efg}$	$2{\cdot}782 \pm 0{\cdot}307^{fg}$	$1{\cdot}78 \pm 0{\cdot}100^{ab}$
(b) F7	Flav.	1	V	+	46	$260{\cdot}7 \pm 28{\cdot}709^{d}$	$124{\cdot}0 \pm 12{\cdot}728^{c}$	$0{\cdot}64 \pm 0{\cdot}113^{def}$	$2{\cdot}312 \pm 0{\cdot}409^{ef}$	$0{\cdot}79 \pm 0{\cdot}103^{a}$
(b) F8	Izy.	1	V	+	54	$166{\cdot}4 \pm 7{\cdot}637^{c}$	$4{\cdot}1 \pm 2{\cdot}546^{a}$	$1{\cdot}02 \pm 0{\cdot}099^{gh}$	$3{\cdot}139 \pm 0{\cdot}305^{fgh}$	$1{\cdot}05 \pm 0{\cdot}134^{ab}$
(b) F9	Alc.	1	2 V	+	46	$182{\cdot}6 \pm 9{\cdot}899^{c}$	$115{\cdot}7 \pm 7{\cdot}495^{c}$	$0{\cdot}75 \pm 0{\cdot}099^{efg}$	$2{\cdot}710 \pm 0{\cdot}358^{fg}$	$1{\cdot}96 \pm 0{\cdot}756^{ab}$
(c) F10	Flav.	1	2 V	+	46	$278{\cdot}7 \pm 20{\cdot}930^{d}$	$287{\cdot}8 \pm 9{\cdot}899^{e}$	$0{\cdot}27 \pm 0{\cdot}099^{abc}$	$0{\cdot}976 \pm 0{\cdot}358^{abc}$	–
(c) F11	Izy.	1	2 V	+	46	$170{\cdot}4 \pm 8{\cdot}061^{c}$	$183{\cdot}7 \pm 2{\cdot}263^{d}$	$0{\cdot}35 \pm 0{\cdot}057^{bcd}$	$1{\cdot}265 \pm 0{\cdot}204^{cde}$	–
(c) F12	Alc.	12	V	+	46	$194{\cdot}9 \pm 4{\cdot}808^{c}$	$122{\cdot}5 \pm 5{\cdot}940^{c}$	$1{\cdot}04 \pm 0{\cdot}085^{gh}$	$3{\cdot}758 \pm 0{\cdot}307^{gh}$	$2{\cdot}39 \pm 0{\cdot}157^{b}$
(c) F13	Flav.	12	V	+	54	$272{\cdot}9 \pm 28{\cdot}991^{d}$	$159{\cdot}2 \pm 11{\cdot}455^{d}$	$1{\cdot}30 \pm 0{\cdot}071^{h}$	$4{\cdot}001 \pm 0{\cdot}218^{h}$	$2{\cdot}01 \pm 0{\cdot}611^{ab}$
(c) F14	Izy.	12	V	+	54	$188{\cdot}8 \pm 13{\cdot}435^{c}$	$66{\cdot}8 \pm 6{\cdot}364^{b}$	$1{\cdot}26 \pm 0{\cdot}113^{h}$	$3{\cdot}878 \pm 0{\cdot}348^{h}$	$1{\cdot}73 \pm 0{\cdot}126^{ab}$

Run	Enzyme	Time EH (h)	*V*	Nut.	$Lactose_{t=0}$(g/l)	$Lactose_{t=f}$(g/l)	$LA_{t=0}$(g/l)	$LA_{t=f}$(g/l)	Q_{LA} (g/l·h)	$Y_{LA/Lactose}$(g/g)	$AcH_{t=f}$(g/l)
(a) F1	–	–	–	–	$48{\cdot}5 \pm 1{\cdot}697^{cd}$	$48{\cdot}1 \pm 1{\cdot}273^{a}$	$0{\cdot}39 \pm 0{\cdot}042^{a}$	$0{\cdot}39 \pm 0{\cdot}085^{a}$	–	–	$0{\cdot}63 \pm 0{\cdot}023^{h}$
(a) F2	–	–	–	+	$48{\cdot}3 \pm 2{\cdot}546^{cd}$	0	$0{\cdot}39 \pm 0{\cdot}028^{a}$	$33{\cdot}5 \pm 0{\cdot}566^{c}$	$0{\cdot}467 \pm 0{\cdot}031^{ab}$	$0{\cdot}69 \pm 0{\cdot}085^{bcd}$	$4{\cdot}3 \pm 0{\cdot}042^{ae}$
(b) F3	Alc.	1	V	–	$49{\cdot}0 \pm 1{\cdot}131^{d}$	$47{\cdot}6 \pm 0{\cdot}849^{a}$	$0{\cdot}40 \pm 0{\cdot}014^{a}$	$1{\cdot}3 \pm 0{\cdot}283^{a}$	$0{\cdot}012 \pm 0{\cdot}003^{d}$	$1{\cdot}9 \pm 0{\cdot}410^{e}$	0
(b) F4	Flav.	1	V	–	$48{\cdot}7 \pm 1{\cdot}273^{d}$	$28{\cdot}0 \pm 1{\cdot}838^{b}$	$0{\cdot}47 \pm 0{\cdot}057^{a}$	$17{\cdot}0 \pm 1{\cdot}414^{b}$	$0{\cdot}232 \pm 0{\cdot}013^{e}$	$0{\cdot}80 \pm 0{\cdot}049^{cd}$	$2{\cdot}9 \pm 0{\cdot}157^{c}$
(b) F5	Izy.	1	V	–	$47{\cdot}9 \pm 2{\cdot}263^{cd}$	$47{\cdot}6 \pm 2{\cdot}121^{a}$	$0{\cdot}50 \pm 0{\cdot}014^{a}$	$0{\cdot}54 \pm 0{\cdot}099^{a}$	$0{\cdot}001 \pm 0{\cdot}000$	$0{\cdot}14 \pm 0{\cdot}004^{ab}$	0
(b) F6	Alc.	1	V	+	$47{\cdot}4 \pm 2{\cdot}121^{cd}$	0	$0{\cdot}44 \pm 0{\cdot}014^{a}$	$39{\cdot}6 \pm 1{\cdot}556^{de}$	$0{\cdot}551 \pm 0{\cdot}011^{ac}$	$0{\cdot}83 \pm 0{\cdot}021^{cd}$	$5{\cdot}0 \pm 0{\cdot}141^{ab}$
(b) F7	Flav.	1	V	+	$46{\cdot}4 \pm 2{\cdot}404^{cd}$	0	$0{\cdot}47 \pm 0{\cdot}071^{a}$	$39{\cdot}3 \pm 0{\cdot}990^{de}$	$0{\cdot}546 \pm 0{\cdot}017^{ac}$	$0{\cdot}84 \pm 0{\cdot}021^{d}$	$5{\cdot}0 \pm 0{\cdot}283^{ab}$
(b) F8	Izy.	1	V	+	$47{\cdot}1 \pm 1{\cdot}131^{cd}$	0	$0{\cdot}49 \pm 0{\cdot}028^{a}$	$41{\cdot}7 \pm 1{\cdot}980^{e}$	$0{\cdot}580 \pm 0{\cdot}023^{c}$	$0{\cdot}87 \pm 0{\cdot}007^{d}$	$4{\cdot}0 \pm 0{\cdot}141^{de}$
(b) F9	Alc.	1	2 V	+	$47{\cdot}3 \pm 0{\cdot}566^{cd}$	0	$0{\cdot}40 \pm 0{\cdot}099^{a}$	$38{\cdot}8 \pm 0{\cdot}566^{de}$	$0{\cdot}541 \pm 0{\cdot}013^{ac}$	$0{\cdot}81 \pm 0{\cdot}017^{cd}$	$5{\cdot}3 \pm 0{\cdot}170^{b}$
(c) F10	Flav.	1	2 V	+	$46{\cdot}8 \pm 0{\cdot}990^{cd}$	$47{\cdot}4 \pm 0{\cdot}14^{a}$	$0{\cdot}51 \pm 0{\cdot}028^{a}$	$0{\cdot}61 \pm 0{\cdot}170^{a}$	$0{\cdot}002 \pm 0{\cdot}001$	$0{\cdot}24 \pm 0{\cdot}321^{abc}$	$3{\cdot}4 \pm 0{\cdot}212^{cd}$
(c) F11	Izy.	1	2 V	+	$45{\cdot}7 \pm 2{\cdot}121^{bcd}$	$45{\cdot}1 \pm 1{\cdot}414^{a}$	$0{\cdot}49 \pm 0{\cdot}099^{a}$	$0{\cdot}51 \pm 0{\cdot}028^{a}$	$0{\cdot}001 \pm 0{\cdot}001$	$0{\cdot}03 \pm 0{\cdot}002^{a}$	$2{\cdot}0 \pm 0{\cdot}113^{i}$
(c) F12	Alc.	12	V	+	$41{\cdot}9 \pm 0{\cdot}566^{abc}$	0	$2{\cdot}9 \pm 0{\cdot}424^{b}$	$36{\cdot}0 \pm 1{\cdot}131^{cd}$	$0{\cdot}467 \pm 0{\cdot}013^{ab}$	$0{\cdot}79 \pm 0{\cdot}007^{cd}$	$6{\cdot}3 \pm 0{\cdot}170^{f}$
(c) F13	Flav.	12	V	+	$39{\cdot}0 \pm 1{\cdot}13^{a}$	0	$3{\cdot}5 \pm 0{\cdot}566^{b}$	$36{\cdot}7 \pm 1{\cdot}838^{cd}$	$0{\cdot}468 \pm 0{\cdot}001^{ab}$	$0{\cdot}85 \pm 0{\cdot}037^{d}$	$7{\cdot}3 \pm 0{\cdot}184^{g}$
(c) F14	Izy.	12	V	+	$39{\cdot}5 \pm 1{\cdot}697^{ab}$	0	$3{\cdot}4 \pm 0{\cdot}707^{b}$	$32{\cdot}5 \pm 1{\cdot}556^{c}$	$0{\cdot}410 \pm 0{\cdot}057^{b}$	$0{\cdot}74 \pm 0{\cdot}082^{cd}$	$6{\cdot}6 \pm 0{\cdot}283^{fg}$

(a) Influence of nutrients addition on crude cheese whey: FI and F2; (b) Influence of enzymatic hydrolysis of whey during 1 h and nutrients addition: F3 to F8; (c) Influence of the enzymatic hydrolysis conditions: F9 to F14.

and 100 rpm, with pH automatically controlled to 6·2 with 5 N NaOH as described by Rodríguez et al. (2012) and Rodríguez-Pazo et al. (2013). Fermentations were carried out working with 1800 ml corresponding: 1615 ml to sterilised cheese whey hydrolysates; 100 ml of nutrients elaborated after resuspending 50 ml freeze-dried and 20 g/l solid vinasses in 100 ml hydrolysed cheese whey; and 85 ml of seed culture used for inoculation.

All fermentations were performed in duplicate and standard deviation of mean values reported in the text.

Kinetic parameters and yields of fermentations

The kinetic parameters and yields of fermentations were calculated at the time where the PLA was maximal. Global volumetric productivity of PLA (Q_{PLA}, g/(L·h)) was calculated as the ratio between the PLA concentration (mg/l) and the fermentation time (h). Phe to PLA yield ($Y_{PLA/Phe}$ g/g) was defined as the ratio between PLA produced ($PLA_{final}-PLA_{t=0}$) and Phe consumed ($Phe_{t=0}-Phe_{final}$) without considering the amount of phenylalanine released in the course of fermentation. Global volumetric productivity of LA (Q_{LA}, g/(L·h)) was calculated as the ratio between the LA concentration (g/l) and the fermentation time (h). Lactose to LA yield ($Y_{LA/Lactose}$, g/g) was defined as the ratio between LA produced ($LA_{final}-LA_{t=0}$) and lactose consumed ($Lactose_{t=0}-Lactose_{final}$).

Antimicrobial effect of CFS

Cell-free supernatants (CFS) were obtained by continuous fermentation of cheese whey hydrolysates supplemented with vinasses using a dilution rate of 0·0207 h^{-1}. The hydrolysates were centrifuged at 2755 ***g*** for 15 min and 10 °C and filter-sterilised using 0·22 µM pore-size membranes to obtain CFS. The bacteriocin activity was determined according to the well-diffusion method described by Rodríguez-Pazo et al. (2013) against *C. piscicola*.

Analytical methods

Total solids in cheese whey were determined by dry weight using 5 g of sample maintained in the oven at 105 °C until constant weight. Total protein concentration was determined by the Pierce Bicinchoninic Acid assay Kit (Thermo Scientific). Fat content in whey (before or after enzymatic hydrolysis with Flavourzyme at 50 °C and 100 rpm during 12 h) was quantified according to ISO 1443. Metals and amino acids were quantified as described Salgado et al. (2009).

Samples were taken during fermentation and centrifuged at 2755 ***g*** for 15 min at 4 °C using a Centrifuge EBA 20. Supernatants were filtered by 0·22 µM pore-size membranes (Millipore) for lactose, lactic acid, acetic acid, Phe and PLA analyses by high-performance liquid chromatographic (HPLC) under the conditions described by Rodríguez-Pazo et al. (2013), while the cells were used for biomass concentration determination. Cells from a known volume of culture media were washed twice with distilled water and centrifuged under the same conditions reported previously. The resulting pellets were oven-dried at 105 °C to constant weight.

Statistical analysis

Mean values of fermentations were submitted to analysis of variance (ANOVA) by the Statistica Software 13.0. They were compared using the Tukey's test at significance level (P) < 0·05, and different letters were used to label values with statistically significant differences among them.

Results and discussion

Optimisation of cheese whey enzymatic hydrolysis

The production of DL-3-Phenyllactic acid (PLA) requires the availability of phenylalanine (Phe) among other compounds in the culture broth, considering that LAB have complex nutrient requirements since they have a limited capacity to synthesise vitamin B and amino acids (de Lima et al. 2010). Hence, the controlled hydrolysis of cheese whey proteins with specific enzymes, could be used to provide a nitrogen source suitable for growth promotion in industrial fermentation, thus eliminating, or reducing, the need for expensive supplements (Galvão et al. 2009).

Using the necessary amount of enzyme to hydrolyse 10 g/l of protein during 1 h (V), flavourzyme showed the higher amounts of free Phe (107·8 mg/l), followed by alcalase (28·9 mg/l) and iZyme (13·5 mg/l), that is at least 3-fold higher than untreated cheese whey content of Phe (4·3 mg/l). An increment in time from 1 to 12 h, increased the concentration of Phe to 120·0, 42·0 and 35·9 mg/l, using respectively flavourzyme, alcalase and iZyme. However, using the double amount of enzyme (2 V) and 1 h of hydrolysis, the Phe concentrations achieved were only 125·8, 29·7 and 17·5 mg/l using respectively flavourzyme, alcalase and iZyme.

Fermentation of crude or enzymatically hydrolysed cheese whey

Crude cheese whey and hydrolysates obtained in previous enzymatic experiments were inoculated with strains of *L. plantarum* and tested for the production of PLA. The growth in absence of a metabolically relevant number of contaminations was double checked by examination of morphology in microscope and visual observation of uniform colonies on agar plates.

Cheese whey without enzymatic hydrolysis (crude cheese whey) was assayed as fermentation broth in absence or presence of the nutrients optimised by Brinques et al. (2010) for *L. plantarum*. During the fermentation of crude cheese whey, in absence of nutrients (F1), Phe was continuously released from the initial 4·3 ± 0·283 mg/l up to 104·3 ± 6·505 mg/l after 78 h (see Table 1). However, PLA was scarcely produced (0·017 ± 0·006 mM) to achieve a volumetric productivity (Q_{PLA}) of only 0·036 ± 0·012 mg/l·h, indicating that the

rate of Phe formation was not enough to fulfil the microorganism requirements for the formation of product. Vermeulen et al. (2006) reported that *Lactobacillus sanfranciscensis* hydrolysed 76–100% of the peptides supplied as dipeptides-containing phenylalanine, thus causing an accumulation of Phe in the course of fermentation. Similarly, *L. plantarum* hydrolysed dipeptides as well, increasing the Phe levels from 0·44 up to 1·38 mM after 72 h. Additionally, these authors also reported that peptide hydrolysis and PLA formation continued when growth had ceased, showing that amino acid conversion is not related to exponential growth but an ongoing process in stationary cells. Using this fermentation broth, lactose was not consumed, and consequently negligible amounts of organic acids were quantified.

The fermentation broth prepared with cheese whey and nutrients (F2) revealed that a considerable proportion of Phe comes from these nutrients, increasing the initial amount of Phe up to 152·9 ± 6·364 mg/l. This supplementary amount of Phe, stimulated the formation of PLA up to 0·93 ± 0·085 mM, and consequently Q_{PLA} to 2·188 ± 0·199 mg/l·h. Besides, from the high value of product yield calculated ($Y_{PLA/Phe}$ = 1·18 ± 0·102 g/g) it can also be concluded that Phe was also released during the process, as it happened in the previous fermentation. In this case, lactose was completely metabolised after 71 h, reaching a lactic acid concentration of 33·5 ± 0·566 g/l (Q_{LA} = 0·467 ± 0·031 g/l·h) and 4·3 ± 0·042 g/l acetic acid.

Considering the complexity of the culture medium, cheese whey hydrolysed during 1 h was assayed in the absence (F3, F4 and F5) or presence (F6, F7 and F8) of nutrients. As it can be seen in Table 1 the tendency was similar to that observed using crude hydrolysates, with increased PLA concentrations after supplementation with nutrients. iZyme, followed by alcalase and flavourzyme showed the highest concentration of PLA, with values of 1·02 ± 0·099, 0·77 ± 0·085 and 0·64 ± 0·113, respectively, in experiments performed after supplementation. With the only exception of hydrolysates obtained with flavourzyme (F4), where lactose was partially consumed to produce 17·0 ± 1·414 g/l lactic acid and 2·9 ± 0·157 g/l acetic acid, the absence of nutrients was unfavourable for fermentation. Conversely, after nutrient addition (F6–F8), lactose was completely consumed, yielding high levels of lactic (39·3 ± 0·990 to 41·7 ± 1·980 g/l) and acetic (4 ± 0·141 to 5 ± 0·283 g/l) acids.

The influence of enzymatic hydrolysis can be inferred comparing F2 (crude cheese whey supplemented with nutrients) with F6, F7 and F8 (cheese whey after enzymatic hydrolysis and supplementation with nutrients). Only a slight increment in PLA concentration was observed in F8 after enzymatic hydrolysis with iZyme, from 0·93 ± 0·085 up to 1·02 ± 0·099 mM. However, in all cases, the time required to achieve the maximal amount of PLA was considerably reduced, from 71 h to only 46–54 h. Therefore, it can be concluded a beneficial effect of enzymatic hydrolysis for the metabolic pathway.

Finally, two strategies were evaluated in order to improve the production of PLA: doubling the amount of enzyme required to hydrolyse 10 g/l of protein (F9, F10 and F11) or increasing the time of hydrolysis to 12 h (F12, F13 and F14).

The first strategy was detrimental resulting in a slight reduction in the amount of PLA (see Table 1), which could be due to an excess of enzyme was harmful, or that the large number of compounds released could inhibit the fermentation. In fact, using the enzyme Flavourzyme (F10), despite the higher value of initial Phe (278·7 ± 20·930 mg/l), the value of Phe at the end of fermentation increased even further to 287·8 ± 9·899 mg/l. Regarding the generation of lactic and acetic acids, no influence was observed using Alcalase (F9), since lactose was entirely depleted, bearing reasonable amounts of lactic acid (38·8 ± 0·566 g/l). However, using Flavourzyme or iZyme, lactose was not consumed at the end of fermentation and consequently no lactic acid was produced.

Conversely, the second strategy was more positive, and therefore the highest concentration of PLA (1·30 ± 0·071 mM) and global volumetric productivity (Q_{PLA} = 4·001 ± 0·218 mg/l·h) were attained in experiment F13 using the necessary amount of enzyme Flavourzyme to hydrolyse 10 g/l of protein, during 12 h. Similar results (PLA = 1·26 ± 0·113 mM; Q_{PLA} = 3·878 ± 0·348 mg/l·h) were achieved using the enzyme iZyme and 12 h of hydrolysis, in spite of starting with a lower amount of Phe (188·8 ± 13·435 mg/l) compared with the 272·9 ± 28·991 mg/l of Phe when using Flavourzyme. The calculated values of product yield also suggested a continuous release of Phe in the course of the fermentation. The results of PLA achieved after enzymatic hydrolysis during 1 h (F6, F7 and F8) or 12 h (F12, F13 and F14) confirmed that the metabolic pathway not only depends on the concentration of the biosynthetic precursor (phenylalanine), since these values are similar independently of the time of hydrolysis, but also supported the hypothesis that during the enzymatic hydrolysis, some compounds that may promote or stimulate the metabolic pathway of Phe into PLA, were also released. In these fermentations, lactose was effectively converted into lactic and acetic acids, according to the data summarised in Table 1.

Characterisation of raw and hydrolysed cheese whey

Raw cheese whey and cheese whey hydrolysed under optimised conditions (Flavourzyme at 50 °C during 12 h and 100 rpm) were characterised and the main parameters summarised in Table 2. The hydrolysis of cheese whey promotes the fractionation of proteins into smaller units. These compounds have more water biding sites, thus increasing the whey solubility (Sinha et al. 2007; Corrêa et al. 2014). This occurrence could explain the decrease in cheese whey total solids in our study from 63·5 ± 0·283 to 54·4 ± 0·460 g/l and proteins from 11·2 ± 0·011 to 8·2 ± 0·022 g/l. The increment of nutrients can be inferred from the higher quantification of amino acids and metals determined after cheese whey hydrolysis (see Table 2). All the amino acids content increased during the hydrolytic process with the only exception of histidine, arginine and proline. Due to the objective of this study, it

Table 2. Characterisation of crude cheese whey and cheese whey after enzymatic hydrolysis (Flavourzyme at 50 °C and 100 rpm during 12 h)

	Before	After	Increment (%)
pH	6·3 ± 0·013	7·0 ± 0·019	
Total solids (g/l)	63·5 ± 0·283	54·4 ± 0·460	−14·3
Lactose (g/l)	48·5 ± 0·207	49·5 ± 0·196	2·1
Lactate (g/l)	0·15 ± 0·165	0·21 ± 0·203	40·0
Protein (g/l)	11·2 ± 0·011	8·2 ± 0·022	−26·8
Fat (g/l)	3·7 ± 0·017	3·7 ± 0·028	0·0
Amino acids (g/l)			
Hydroxyproline	0·01 ± 0·00	0·01 ± 0·00	10·0
Aspartic acid	0·11 ± 0·02	0·16 ± 0·03	43·5
Serine	0·29 ± 0·08	0·36 ± 0·09	25·2
Glutamic acid	0·29 ± 0·07	0·45 ± 0·10	57·0
Glycine	0·05 ± 0·01	0·07 ± 0·02	31·4
Histidine	0·33 ± 0·08	0·19 ± 0·03	−43·4
Taurine	0·03 ± 0·00	0·04 ± 0·01	28·8
Arginine	0·15 ± 0·03	0·15 ± 0·04	−2·6
Threonine	0·18 ± 0·08	0·20 ± 0·09	11·4
Alanine	0·21 ± 0·09	0·27 ± 0·11	24·9
Proline	0·05 ± 0·01	0·05 ± 0·01	−1·2
Tyrosine	0·14 ± 0·04	0·16 ± 0·05	15·0
Valine	0·28 ± 0·08	0·33 ± 0·09	17·4
Methionine	0·16 ± 0·05	0·17 ± 0·04	7·6
Lysine	0·59 ± 0·11	0·74 ± 0·14	25·4
Isoleucine	0·31 ± 0·07	0·35 ± 0·05	11·6
Leucine	0·54 ± 0·14	0·65 ± 0·17	19·2
Phenylalanine	0·15 ± 0·04	0·18 ± 0·08	21·2
Metals (mg/kg)			
K	1·6 ± 0·04	2·0 ± 0·09	25·0
Cu	<1·8	<1·8	–
Zn	<1·0	<1·0	–
Fe	<6·0	<6·0	–
Mn	<1·2	<1·2	–
Ca	292 ± 23	362 ± 34	24·0
Mg	79·2 ± 8	77·5 ± 14	−2·1
Na	456 ± 31	915 ± 56	100·7
Al	<30	<30	–

is particularly noticeable the increment of 21·2% observed with phenylalanine. Regarding the study of metals, after hydrolysis, it was observed a strong increment in the amount of Na, K and Ca. Mg decreased slightly; meanwhile the remaining metals were hardly quantified. Rossini et al. (2009) demonstrated in their work the efficiently of Flavourzyme to hydrolyse the casein molecule. The major concentration of Ca, present in raw cheese, is partially bonded with casein micelles. When this structure is hydrolysed, normally the Ca concentration increased (Gaucheron, 2005).

Fermentation of cheese whey hydrolysates supplemented with vinasses

The solid or the freeze-dried liquid fractions of wine vinasses were evaluated as the only nitrogen source in order to increase the economical potential for larger-scale bioproduction. Salgado et al. (2009) suggested that, in spite of being one of the most common nitrogen sources in a variety of bioprocesses, the high cost of yeast extract impairs the economics, estimating that its value accounts for 38% of the final cost of lactic acid.

Figure 1 depicts the kinetics of Phe and lactose consumption as well as the generation of phenyllactic and lactic acids; meanwhile Table 3 summarises the parameters of fermentation. Using vinasses as a source of nitrogen, the concentration of PLA ranged between 1·15 ± 0·071 and 1·20 ± 0·052 mM, slightly lower to the value of 1·55 ± 0·060 mM achieved with commercial nutrients (used as control). Nevertheless, these values are comparable or superior to those reported by Vermeulen et al. (2006) using *L. sanfranciscensis* or *L. plantarum* (0·25–0·75 mM respectively); Li et al. (2007) using *Lactobacillus* sp. SK007 (0·55 mM); Rodríguez et al. (2012) using *L. acidophilus, Lactobacillus pentosus, L. plantarum, L. rhamnosus,* and *Lactococcus lactis* (0·17–1·38 mM) with the highest value working with *L. plantarum*; Valerio et al. (2004) during the screening of 28 strains (0·02–0·57 mM) or the 13 LAB screened by Cortés-Zavaleta et al. (2014) in culture tubes prepared with fresh sterile MRS broth (0·021–0·275 mM), although in this case, no PLA was quantified with *L. plantarum*.

The product yields oscillated between 0·53 ± 0·014 and 0·68 ± 0·460 g/g in all cases. The most outstanding result could be the fact that using 50 ml freeze-dried vinasses and 20 g/l solid vinasses, the time of fermentation was reduced to only 24 h, thus increasing the Q_{PLA} up to 0·008 ± 0·005 g/l·h and the volumetric rate of Phe consumption (Q_{Phe}) up to 0·0138 ± 0·0003 g/l·h. In contrast, lactose was efficiently converted in lactic acid in all fermentations, with product yields ranging from 0·70 ± 0·020 to 0·93 ± 0·059 g/g, although the higher lactic acid value (40·3 ± 0·778 g/l) was achieved in fermentations carried out using 100 ml freeze-dried vinasses as nutrients (fermentation d). Consequently, vinasses could be employed to overcome one of the major technical hurdles for the development of low-cost culture media that could stimulate the production of natural products. Taking into account not only the higher global volumetric productivities achieved for both lactic acid and PLA when using freeze-dried and solid vinasses, but also considering the higher profit of using both fractions, further experiments were conducted using this combination of vinasses as nutrients in continuous processes.

Continuous fermentation of cheese whey hydrolysates supplemented with vinasses

Finally, the process was scaled up to a 2 L Braun Biostat fermenter operating on continuous. Table 3 shows the results obtained under three steady states reached at selected dilution rates. As it was expected, under the lowest dilution rate assayed (0·0057 h^{-1}), although the maximal amount of PLA concentration was achieved (0·91 ± 0·085 mM), the process rendered a low Q_{PLA} (0·0086 ± 0·0001 g/l·h) and Q_{Phe} (0·0039 ± 0·0002 g/l·h) at a reasonable $Y_{PLA/Phe}$ (0·48 ± 0·007 g/g). The PLA concentration decreased continuously

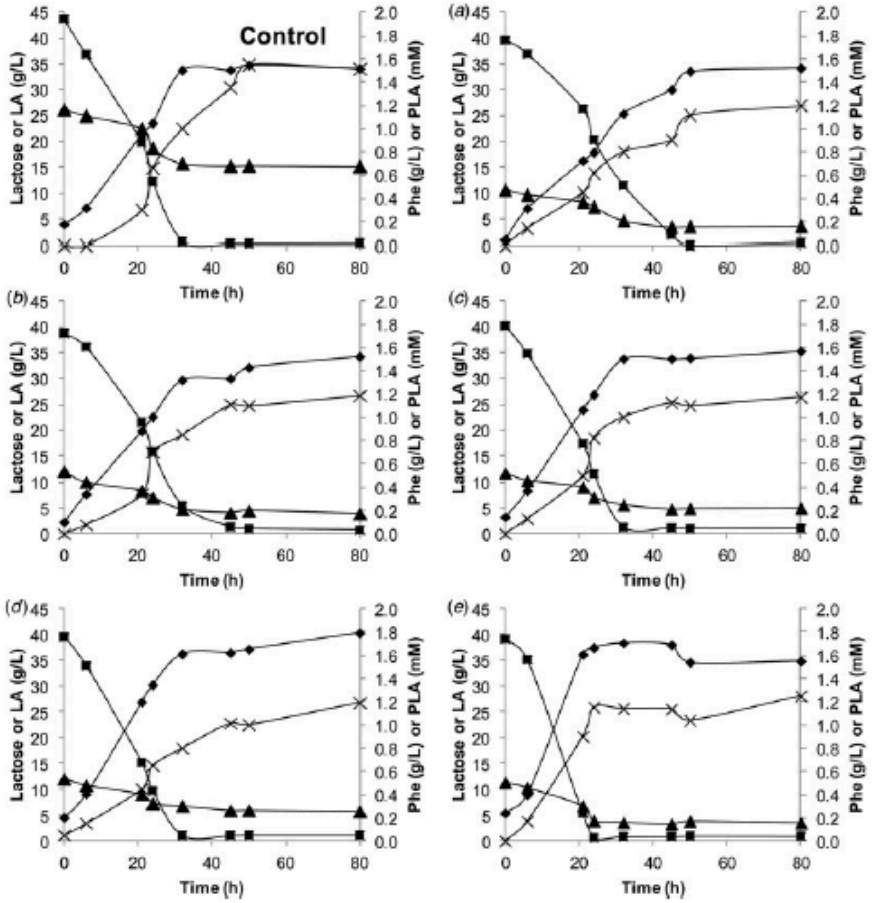

Fig. 1. Kinetics of Phe and lactose consumption, and PLA and lactic acid production using cheese whey hydrolysates as carbon source and vinasses as nitrogen source in fermentations carried out in Erlenmeyer flasks. (*a*) 25 ml freeze-dried vinasses; (*b*) 50 ml freeze-dried vinasses; (*c*) 75 ml freeze-dried vinasses; (*d*) 100 ml freeze-dried vinasses; (*e*) 50 ml freeze-dried vinasses and 20 g/l solid vinasses. Lactose (■); lactic acid (♦); Phe (▲); PLA (×).

when the dilution rate was increased; with the lowest PLA concentration (0·65 ± 0·127 mM) and $Y_{PLA/Phe}$ (0·17 ± 0·036 g/g) achieved under the highest dilution rate assayed (0·0393 h^{-1}). Under this dilution rate, the higher Q_{PLA} (0·0042 ± 0·0008 g/l·h) and Q_{Phe} (0·025 ± 0·0006 g/l·h) were achieved, meaning that it cannot be inferred an optimal value to work with.

Conversely, regarding the conversion of lactose into lactic acid, lactose was completely consumed in all cases (see Table 3); yielding similar lactic acid concentrations (38·4 ± 3·932 to 39·7 ± 1·980 g/l) corresponding to products yields of 0·85 ± 0·161 to 0·88 ± 0·012 g/g. However, an optimum value of Q_{LA} (1·563 ± 0·078 g/l·h) and $Q_{Lactose}$ (0·859 ± 0·460 g/l·h) was achieved under the highest dilution rate, considering that a similar lactic acid concentration was attained in a shorter period of time.

Antimicrobial effect of CFS

Finally, the antimicrobial activity of cell-free supernatants (CFS) was evaluated against *C. piscicola* as indicator microorganism. CFS extracts produced halos with mean values of 12·03 ± 0·50 mm. Commercial lactic acid, PLA and nisin (in the amounts present in CFS) were also evaluated as a control and the mean inhibitory halos depicted in Fig. 2. Using 41 g/l of commercial lactic acid, the inhibitory halo was 6·60 ± 0·63 mm, meaning that the effect of this commercial acid was equivalent to 54·9% of the value obtained with CFS. Conversely, the use of 0·81 mM of commercial PLA had no inhibitory effect. It was necessary to increase the amount of PLA up to 7·5 mM to observe a minimum effect of 0·10 ± 0·05 mm. However, commercial nisin with equivalent concentration (1·25 g/l) increased the inhibitory

Table 3. Summary of values obtained in the fermentation of cheese whey hydrolysates and vinasses under different fermentation conditions: using 250 ml Erlenmeyer flasks or 2 litres Bioreactor operating in continuous under three dilution rates (in h^{-1})

	Erlenmeyer flasks						Bioreactor		
	Control	(a)	(b)	(c)	(d)	(e)	0·0057 h^{-1}	0·0207 h^{-1}	0·0393 h^{-1}
Time (h)	50	80	80	80	80	24	–	–	–
$Phe_{t=0}$ (g/L)	1·2 ± 0·090^{b}	0·47 ± 0·071^{a}	0·54 ± 0·042^{a}	0·52 ± 0·113^{a}	0·53 ± 0·071^{a}	0·51 ± 0·049^{a}	–	–	–
Phe_{final} (g/l)	0·68 ± 0·040^{d}	0·16 ± 0·031^{a}	0·17 ± 0·027^{a}	0·22 ± 0·042^{ab}	0·26 ± 0·035^{abc}	0·18 ± 0·042^{a}	0·32 ± 0·042^{bc}	0·37 ± 0·014^{c}	0·36 ± 0·014^{bc}
$PLA_{t=0}$ (mM)	0	0	0	0	0	0	–	–	–
PLA_{final} (mM)	1·55 ± 0·060c	1·20 ± 0·052a	1·19 ± 0·082a	1·17 ± 0·085a	1·19 ± 0·092a	1·15 ± 0·071a	0·91 ± 0·085ab	0·81 ± 0·026b	0·65 ± 0·127b
Q_{PLA} (g/l·h)	0·0052 ± 0·0000^{b}	0·0025 ± 0·0001^{a}	0·0025 ± 0·0002^{a}	0·0024 ± 0·0002^{a}	0·0025 ± 0·0002^{a}	0·0080 ± 0·0005^{d}	0·00086 ± 0·0001^{c}	0·00028 ± 0·0001^{a}	0·0042 ± 0·0008^{b}
Q_{Phe} (g/l·h)	0·0104 ± 0·0010^{c}	0·0039 ± 0·0005^{a}	0·0046 ± 0·0002^{a}	0·0038 ± 0·0009^{a}	0·0034 ± 0·0004^{a}	0·0138 ± 0·0003^{b}	0·0039 ± 0·0002^{a}	0·013 ± 0·0003^{b}	0·025 ± 0·0006^{d}
$Y_{PLA/Phe}$ (g/g)	0·53 ± 0·029^{a}	0·64 ± 0·055^{ab}	0·53 ± 0·014^{a}	0·66 ± 0·109^{ab}	0·74 ± 0·040^{b}	0·58 ± 0·023^{ab}	0·22 ± 0·007^{c}	0·21 ± 0·002^{c}	0·17 ± 0·036^{c}
$Lactose_{t=0}$ (g/l)	43·7 ± 2·107^{a}	39·6 ± 2·263^{a}	38·7 ± 2·404^{a}	40·2 ± 0·085^{a}	39·5 ± 2·828^{a}	39·1 ± 1·909^{a}	45·1 ± 3·507^{a}	45·1 ± 1·612^{a}	45·1 ± 1·612^{a}
$Lactose_{final}$ (g/l)	0	0·55 ± 0·403^{a}	0·7 ± 0·240^{a}	1·01 ± 1·089^{a}	1·15 ± 0·523^{a}	0·63 ± 0·297^{a}	0	0	0
$LA_{t=0}$ (g/l)	4·0 ± 0·813^{ab}	1·1 ± 0·806^{c}	2·3 ± 0·085^{ac}	3·3 ± 0·849^{abc}	4·6 ± 0·099^{ab}	5·4 ± 0·778^{b}	–	–	–
LA_{t} (g/l)	34·8 ± 1·414^{a}	34·2 ± 1·131^{a}	34·2 ± 1·838^{a}	35·3 ± 1·838^{a}	40·3 ± 0·778^{a}	37·5 ± 1·838^{a}	38·4 ± 3·932^{a}	38·8 ± 3·253^{a}	39·7 ± 1·980^{a}
Q_{LA} (g/l·h)	0·616 ± 0·012^{c}	0·414 ± 0·024^{a}	0·399 ± 0·022^{a}	0·400 ± 0·012^{a}	0·446 ± 0·011^{a}	1·338 ± 0·044^{e}	0·219 ± 0·022^{b}	0·803 ± 0·067^{d}	1·563 ± 0·078^{f}
$Q_{Lactose}$ (g/l·h)	0·875 ± 0·042^{b}	0·491 ± 0·023^{a}	0·475 ± 0·027^{a}	0·490 ± 0·013^{a}	0·479 ± 0·042^{a}	1·603 ± 0·067^{d}	0·257 ± 0·020^{c}	0·932 ± 0·033^{b}	1·774 ± 0·063^{e}
$Y_{LA/Lactose}$ (g/g)	0·70 ± 0·020^{a}	0·84 ± 0·009^{a}	0·84 ± 0·002^{a}	0·82 ± 0·046^{a}	0·93 ± 0·059^{a}	0·83 ± 0·007^{a}	0·85 ± 0·161^{a}	0·86 ± 0·041^{a}	0·88 ± 0·012^{a}

Phe: phenylalanine; PLA: DL-3-Phenyllactic acid; Q_{PLA}: global volumetric productivity of PLA; Q_{Phe}: volumetric rate of Phe consumption; $Y_{PLA/Phe}$: Phe to PLA yield, calculated as $(PLA_{final}–PLA_{t=0})/(Phe_{t=0}–Phe_{final})$ without considering the amount of phenylalanine released in the course of fermentation; LA: lactic acid; Q_{LA}: global volumetric productivity of LA; $Q_{Lactose}$: volumetric rate of lactose consumption; $Y_{LA/Lactose}$: Lactose to LA yield. (a) 25 ml freeze-dried vinasses; (b) 50 ml freeze-dried vinasses; (c) 75 ml freeze-dried vinasses; (d) 100 ml freeze-dried vinasses; (e) 50 ml freeze-dried vinasses and 20 g/l solid.

Different letters mean statistically significant differences among values in the same line ($P < 0·05$).

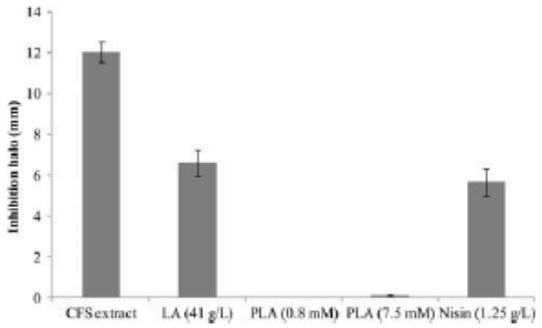

Fig. 2. Halos, expressed as a mean of three replications, induced by the cell-free supernatant of *Lactobacillus plantarum* CECT-221, commercial lactic acid (41 g/l), PLA (0·8 or 7·5 mM) or nisin (1·25 g/l), against the indicator microorganism *Carnobacterium maltaromaticum*.

halo up to 5·65 ± 0·65 mm. These results pointed out the contribution of all the compounds present in the CFS.

Although Lavermicocca et al. (2003) and Prema et al. (2008) found that PLA produced by *L. plantarum* showed inhibitory action against different species of moulds, other authors reported that the high minimum inhibitory concentration for commercial PLA is in the range of 3·01–36·10 mM against some food spoilage moulds. These values suggest that PLA cannot be considered the only compound related with the antifungal potential and that synergistic effects may exist among PLA and other products obtained during the metabolism of LAB (Cortés-Zavaleta et al. 2014). In this way, Tirloni et al. (2014) proposed that the antagonistic ability of LAB as biopreservants can be explained by the competition for nutrients and through the production of antimicrobial compounds such as bacteriocins, reuterin, organic acids (mainly acetic and lactic acids), carbon dioxide, diacetyl, ethanol, hydrogen peroxide and enzymes. In this context, Schwenninger et al. (2008) in addition to PLA also identified by chromatography and mass spectrometry the presence of propionic, acetic, and lactic acids, 2-pyrrolidone 5-carboxylic acid, hydroxyphenyllactic acid, and succinic acid during the co-culture of *Lactobacillus paracasei* and *Propionibacterium jensenii* in supplemented whey permeate medium.

In conclusion, cheese whey can be efficiently enzymatically hydrolysed to improve the availability of phenylalanine. Hydrolysates supplemented with vinasses can be fermented by *Lactobacillus plantarum* CECT-221 in batch or continuous fermentations using 2 litres Bioreactors. Cell-free supernatants showed antimicrobial activity although the results suggested that PLA was not the main metabolite responsible, being more important the contribution of lactic acid and bacteriocins present in the CFS.

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APPENDIX C – Published article in collaboration: Influence of probiotic ingredients on the growth kinetics and bacteriocin production of *Lactococcus lactis.*

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Influence of Prebiotic Ingredients on the Growth Kinetics and Bacteriocin Production of *Lactococcus lactis*

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Prebiotic ingredients are increasingly added to food preparations containing probiotics in order to enhance probiotic survival and growth. Bacteriocins produced by *Lactococcus lactis* (nisin) have great advantages as a food additive, such as heat stability, nontoxicity and sensitivity to digestive proteases. In this context, the aim of this study was to evaluate the influence of prebiotic ingredients on the fermentation kinetics and antimicrobial activity by *Lactococcus lactis* subsp. *lactis* CECT 4434 against *Lactobacillus sakei*. *Lc. lactis* was cultivated in MRS medium (Man, Rogosa and Sharpe) supplemented with or without (control) fructooligosacharides (MRS+FOS), polydextrose (MRS+PD) and inulin (MRS+IN). All cultivations were carried out in shaken flasks at 30 °C with agitation speed of 100 rpm. Samples were collected for analyses (biomass, specific growth rate, generation time and antimicrobial activity) every two hours during the 48 h of cultivation. The exponential growth phase of *Lc. lactis* subsp. *lactis* CECT 4434 occurred at intervals of 2-8 h for all runs (MRS, MRS+FOS, MRS+PD e MRS+IN). The use of all prebiotic ingredients increased cell biomass, specific growth rate and consequently decreased generation time when compared with control (only MRS). Antimicrobial activity of nisin produced by *Lc. lactis* subsp. *lactis* CECT 4434 was detected against *Lactobacillus sakei* strain.

1. Introduction

Recently, the influence of prebiotic ingredients over the cellular growth of lactic acid bacteria (LAB) has sparked an increased interest due to their properties as a functional food and their beneficial effects to the health and well-being of those ingesting them.

Functional dairy products contain probiotic microorganisms belonging to the *Lactobacillus, Bifidobacterium, Streptococcus* and *Saccharomyces* genera. These products have been historically defined as food containing live microorganisms that improve the host's health through the improvement of the microbiotic balance in the human intestinal tract (Tamine, Saarela, Sondergaard, Mistry & Shad, 2005). By definition, probiotics, including some species of *Lactococcus*, are known as living microorganisms which, when used in adequate quantities, provide benefits to the host's health due to being capable of surviving the trip through the upper digestive tract, adhering to intestinal cells and contributing to intestinal balance (FAO/WHO, 2002; Martinez et al., 2013).

Prebiotics are non-digestible hydrocarbons that, through an absorption process, resist hydrolysis in the upper gastrointestinal tract of humans (Mattila-Sandholm et al., 2002; Apolinário et al., 2014). When consumed in adequate amounts, these ingredients reach the colon still intact and improve the modulation and composition of the intestinal microbiota, providing benefits to the host's health and, therefore, of the users (Saad et al., 2013; Roberfroid, 2007; Roberfroid et al., 2010).

Rastall (2010) studied the applications and the industrialization of prebiotics and reported that, although several hydrocarbons are marketed as prebiotics throughout the world, the most prominent ones are the

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inulin, the fructooligosacharides (FOS) and the galactooligosacharides (GOS). Those are, therefore, the most widely studied hydrocarbons in human clinical tests.
Previous studies have assessed the influence of several prebiotics in the growth and acidification of pure probiotic cultures and co-cultures in skimmed fermented milk. These studies reported that the addition of inulin significantly reduces the fermentation time and increases the viability of probiotic bacteria (Oliveira et al., 2011).
In order to achieve a better understanding of the functional activity of prebiotics over probiotic bacteria, it is necessary to develop better symbiotic preparations. The success in the establishment of the necessary symbiotic condition is closely related to the compatibility between the chosen components, that is to say, between the prebiotic ingredients and the probiotic bacteria (Mei et al., 2011; Pranckute et al., 2014, Muñoz et al., 2012). The authors suggest that strains of *Lactobacillus* sp. and *Lactococcus* sp. may be useful as probiotic bacteria when used together with prebiotics (palatinose, inulin and α-cyclodextrin) so as to create a symbiotic development, which could control not only the grown of beneficial bacteria in the gastrointestinal tract, but also their antibacterial activity.
Under the light of this information, this study aims at assessing the influence of several prebiotic ingredients FOS, polydextrose and inulin over the cellular growth of the *Lactococcus lactis* CECT 4434 bacterial strain and evaluate the growth kinetics parameters (μ_{max} e t_g) when cultivated in MRS medium (Man, Rogosa and Sharpe) supplemented or not (control) with fructooligossacharides (MRS+FOS), polydextrose (MRS+PD) and inulin (MRS+IN). The antimicrobial capacity of the bacteriocin produced by *Lc. lactis* was also evaluation in contrast to the bioindicator strain *Lactobacillus sakei*.

2. Material and methodology

2.1 Microbial culture and growth medium

The strain of *Lactococcus lactis* subsp. *lactis* CECT 4434, obtained from the Spanish Cultivation Collection (CECT), was cultivated in MRS medium (Man, Rogosa and Sharpe) supplemented or not (control) with fructooligosacharides (MRS+FOS), polydextrose (MRS+PD) and inulin (MRS+IN). The MRS growth medium contains 20 g/L of polydextrose. For the formulation of the MRS+FOS, MRS+PD and MRS+IN, the dextrose polysacharide was substituted respectively by 20 g/L of fructooligosacharides, polydextrose and inulin.

2.2 Inoculum preparation and cultivation conditions

The inoculum was prepared with the addition of 100 µL of the stock culture in a 250 mL Erlenmeyer flask containing 100 mL of MRS supplemented with one of the hydrocarbons. The flasks were then incubated in a shaker at 100 rpm and 30°C until a cellular concentration of 0.8 – 0.9 D.O. units at 600 nm.
The cultivation of *Lc. lactis* CECT 4434 was conducted with the addition of 10% (v/v) of the inoculum in 250 mL Erlenmeyer flasks containing 100 mL of MRS supplemented individually with the prebiotic ingredient under analysis. The flasks were then incubated at a shaker at 100 rpm and 30°C for 48 hours. Samples were collected every 2 hours for a period of 12 hours and then at intervals of 12 hours, with a total cultivation time of 48 hours.

2.3 Analysis Procedures

In the monitoring process of the cellular growth, the relationship between D.O. (600 nm) and dry mass (mL) was observed after filtration of the growth medium through a 0.22 µm membrane (Millipore). Through this relationship, a calibration curve was obtained, represented by the following equation (1)

$$y = 2.0076 * x + 0.00182 \quad (1)$$

2.4 Growth Kinetics

The growth kinetics for *Lc. lactis* CECT 4434 was investigated during the fermentation, both in the absence of prebiotics (control) and in their presence. The maximum specific speed for the growth (μ_{max}) was calculated during the exponential growth phase through the following equation (2).

$$\mu max = \frac{1}{(t_2 - t_1)} \ln \frac{X_2}{X_1} \quad (2)$$

with X_2 and X_1 representing respectively the dry mass values, in (g/L), at t_1 and t_2.

The generation time was determined through equation (3).

$$t_g = \ln 2 / \mu max \quad (3)$$

2.5 Determination of antimicrobial activity

For detection of bacteriocin activity, samples were centrifuged at 16,000 *g* at 4 °C for 10 minutes. The pH of the supernatant was neutralized to 6.0 – 6.5 using 1 M NaOH in order to eliminate the action of organic acids. In addition, the supernatant was submitted to 80 °C for 10 minutes to eliminate possible proteases. After treatment of the supernatant, it was tested against the strains *L. sakei* ATCC 15521 to evaluate its antimicrobial activity. This test was performed by the agar diffusion assay, in which 10 µL of the indicator strain were transferred to a Petri dish containing 15 mL of MRS agar (Difco, Detroit, MI, USA). Once solidified, 10 µL of supernatant were pipetted on the agar surface. The plates were incubated at 30 °C for 18 to 24 hours and after this period it was possible to observe zones of inhibition.

3. Results and discussion

In the monitoring process of the cellular growth, the relationship between D.O. (600 nm) and dry mass (mL) was observed after filtration of the growth medium through a 0.22 µm membrane (Millipore).

The results shown at Figure 1 enable a comparison between the cellular growth curves of *Lc. lactis* CECT 4434, cultivated in MRS medium supplemented or not (control) with fructooligosacharides (MRS+FOS), polydextrose (MRS+PD) and inulin (MRS+IN).

Upon analyzing the growth curves regarding the cellular concentration, a behavior similar may be observed in terms of *Lc. lactis* CECT 4434 biomass formation for MRS (control), (MRS+FOS) and (MRS+PD) growth media, with values obtained at the tenth hour respectively of 0.94, 0.88 and 0.86 (g/L). On the other hand, under a prebiotic supplement, such as with (MRS+FOS) and (MRS+PD), there was an increase on the formation of biomass starting at 10 hours in contrast with MRS medium with no supplement.

It is evident that the cultivation of *Lc. lactis* CECT 4434 supplemented with inulin (MRS+IN) presented influence over the increase in the biomass production during the 48 hour cultivation. The cellular concentration values of *Lc. lactis* CECT 4434 with inulin as a supplement (MRS+IN) and with no supplement (control) were 1.48 and 0.98 (g/L) respectively.

These results highlight that the biomass production for *Lc. lactis* CECT 4434 is higher when supplemented with inulin, corresponding to the studies conducted by Oliveira *et al.* (2011), Oliveira *et al.* (2012) and Likotrafiti *et al.* (2014), which reported similar results regarding the capacity to increase the cellular count of probiotic microorganisms in cultivations supplemented with inulin.

In addition, in recent study, Kondepudi *et al.* (2012) demonstrated that prebiotic oligosaccharides, such as FOS and GOS may be used as a promising culture medium to optimize the production of probiotic *Bifidobacterium* species. We also observed an increase in the concentration of the *B. breve*, *B. lactis* and *B. longum* biomass, as well as of some BAL, for instance, the *Lactococcus lactis*. The latter showed antimicrobial activity against *Clostridium difficile*.

The effect of the FOS metabolism, extracted from corn silage and molasses, by *Lactobacillus* strains, in the production of bacteriocin, was investigated by Muñoz *et al.* (2012). In this study, it was confirmed that the different lactic acid bacteria LAB, for instance, *Enterococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc* were able to use the FOS determined by the count of viable cells (CFU) successfully. An increase in viable cells count of BAL in the MRS growing medium, supplemented with FOS, was verified, unlike MRS culture medium without supplementation (control), which had a lower score.

Makelainen *et al.* (2010) evaluated the ability of different prebiotics, such as FOS and xylooligosaccharides (XOS), as potential candidates for carbon source to probiotic bacteria of the genres *Bifidobacterium* and *Lactobacillus*, as well as their spectrum of action to possible intestinal microbe pathogens, such as *Eubacterium*, *Bacteroides*, *Clostridium*, *Escherichia coli*, *Salmonella* and *Staphylococcus* in pure cultures. These authors could identify bifidobacteria that had preference for oligosaccharides, thus showing that the culture medium containing FOS and XOS, which have different degrees of polymerisation (DP) showed satisfactory fermentation, especially in strains of *B. lactis* and *B. adolescentis*, the *Lactobacillus*, in their turn, did not metabolize XOS efficiently. The same authors showed, in research published in 2010, that the FOS and GOS were fermented by a wide range of tested microorganisms, especially the *Bifidobacterium* and *Lactobacillus*, denoting the non-selectivity of FOS in pure culture studies. The researchers emphasized that the FOS are not selective only to the genera considered beneficial, but also to other intestinal micro-organisms, such as *Bacteroides spp.*, *Clostridium spp.*, and *Eubacterium spp*.

Mei *et al.* (2011) attributed the growth of probiotic bacteria to the influence of the FOS, which is directly related to the composition of FOS, like the β(2→1) fructans of short chains, once these greatly support the growth of probiotic microorganisms, when compared to the longer-chained β(2→1) fructans. Therefore, the authors could confirm that all β(2→1) fructans with glycosidics links are bifidogenic.

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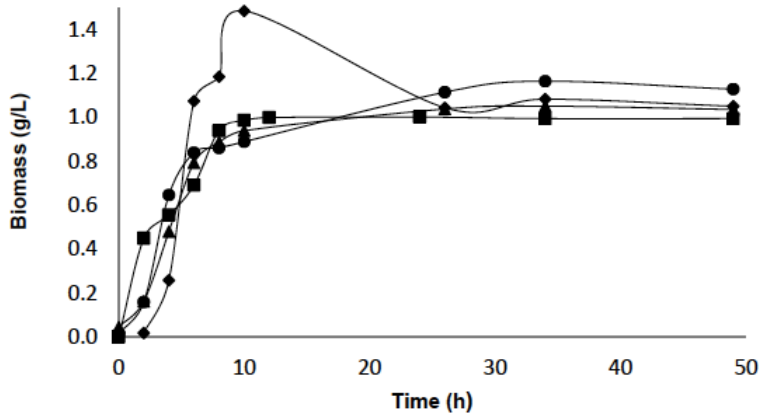

Figure 1. Comparison between the cellular growth curves for Lactococcus lactis subsp. lactis CECT 4434 in MRS medium (control: ■) supplemented with (20g/L) of polydextrose (MRS+PD: ●), (20g/L) of fructooligosacharides (MRS+FOS: ▲) and (20g/L) inulin (MRS+IN: ◆).

Upon observing the cellular concentration of *Lc. lactis* CECT 4434 cultivated in (MRS+IN) as a function of time, it is possible to observe a potential growth during the first 8 hours, reaching the peak biomass production at the eighth hour, with a dry mass value of 1.48 (g/L). Between 8 and 10 hours, the strain of *Lc. lactis* CECT 4434 presented a decrease in cellular production, reaching a dry mass value of 1.04 (g/L), and then presenting stability in cellular growth until 48 hours. These results are in accordance with a recent study conducted by Kassim *et al.* (2014) regarding the effect of chicory leaves, which contain inulin, as a growth medium for cultivating microorganisms from the *Lactococcus* and *Bifidobacterium* genera. The authors have identified a direct relation between the microorganisms' growth, especially those of the *Lactococcus* genus, in a growth medium containing inulin. Similar results have been reported by Oliveira *et al.* (2012), with inulin being among the best functional prebiotic ingredients for preparation of probiotic fermented milk, both for pure cultures and for binary co-cultures. In this study, the authors reported that the addition of inulin significantly reduced the time needed for fermentation, presenting higher biomass growth and an increase in the levels of diacetyl, acetone, lactic acid and acetic acid in both cultures (pure and/or co-culture).
Mei *et al.* (2011) have based themselves on different linear chain compositions of FOS and inulin so as to assess how those are used by different probiotic strains. Therefore, the authors used chicory and oat as a source for FOS and inulin, observing that the twelve probiotic strains tested, from the *Bifidobacterium, Lactobacillus* and *Pediococcus* genera, were capable of metabolizing FOS and inulin so as to optimize the biomass production of these strains.

3.1. Effect of the addition of different prebiotics in the cellular growth kinetics of *Lactococcus lactis subsp. lactis* CECT 4434.

The data for maximum specific speed for growth (μ_{max}) and the generation times (t_g) for *Lc. lactis* CECT 4434 in MRS medium supplemented or not with prebiotics.

Table 1. Values for maximum specific velocity for growth (μ_{max}) for Lactococcus lactis subsp. lactis and generation time (t_g) using MRS medium in the presence of polydextrose, fructooligosacharides and inulin.

Growth media*	Maximum specific speed for growth (μ_{max}) h^{-1}	t_g(h)
MRS	0.13±0.02[a]	2.66±0.08[d]
MRS + FOS	0.48±0.02[b]	1.42±0.04[c]
MRS + PD	0.94±0.03[c]	0.75±0.05[b]
MRS + IN	1.01±0.03[d]	0.68±0.06[c]

Values followed by the same letter in the same column are not different from each other, with probability of 5% by Tukey's test. *MRS (*Man, Rogosa and Sharpe*); PD (poydextrose); FOS (fructooligosacharides) and IN (inulin)

It is possible to observe that the maximum specific speed for growth (μ_{max}) in MRS medium and no supplement (control) was 0.13 h^{-1}, i.e. the lowest value among the tests, while the growth media supplemented with 20g/L of fructooligosacharides (MRS+FOS), polydextrose (MRS+PD) and inulin (MRS+IN) presented μ_{max} values of 0.48, 0.94 and 1.01 h^{-1}, respectively. These results showed that the addition of prebiotic ingredients to the MRS medium in combination with the probiotic strain *Lc. lactis* CECT 4434, positively influenced the metabolism, not only increasing the biomass concentration, but also favoring higher values for growth specific speed, especially in the case of inulin. A similar result was observed by Oliveira *et al.* (2012), Sims et al. (2014) and Saad et al. (2013), who studied the symbiotic interaction of inulin as an stimulating factor for the growth of probiotic microorganisms.

Upon analyzing the generation time (t_g) for *Lc. lactis* CECT 4434 in a MRS medium supplemented or not (control) with polydextrose (MRS+PD), fructoligosacharides (MRS+FOS) and inulin (MRS+IN), it is possible to highlight that t_g is inversely proportional to a μ_{max}, that is to say, the lower the growth specific speed, the higher is the generation time for the microorganism in question. The strain of *Lc. lactis* CECT 4434 cultivated in MRS (control) multiplied every 2.65 h. On the other hand, in a MRS medium supplemented with inulin (MRS+IN), the strain multiplied every 0.68 h. These results indicate that the addition of prebiotics, specially polydextrose and inulin, reduced the generation time for the probiotic strain in contrast to the control.

3.2. Bacteriocin antimicrobial activity

Upon testing the antimicrobial activity of the supernatant obtained by treating the growth medium for *Lc. lactis* CECT 4434 in comparison with *L. sakei* ATCC 15521, a formation of inhibition zones which varied as a function of time was observed. The production of bacteriocin begins within 6 hours of cultivation, generating halos of 12.55 mm in diameter (average value obtained in MRS medium). This phase of the cultivation includes the exponential phase for the strain *Lc. lactis* CECT 4434. After 10 hours, the inhibition zones diameter increase to 15.55 mm, but decrease to 14.85 mm at 16 hours, probably due to a peptidic instability caused by the organic acids produced by the strain in question. The values obtained with the addition of prebiotics to MRS medium were on average 12% lower compared to the control (without supplementation). Gomes *et al.* (2012) observed a reduction of bacteriocin activity by approximately 50% and 62% when the inulin and oligofructose were added to MRS medium. On the other hand, Chen et al. (2007) reported that the addition of FOS and trehalose increased the bacteriocin production by *Lactococcus lactis* ssp. *lactis* C101910.

4. Conclusion

The different prebiotic ingredients used as a growth medium for *Lc. lactis* CECT 4434 presented a positive influence over the growth of microbial biomass. With the fermentation process, it was possible to observe that the prebiotic ingredients foster cellular growth of *Lc. lactis* CECT 4434.

On the different tests conducted, it is possible to observe that the prebiotic ingredients, especially inulin, provided an increase in the maximum specific speed for growth (μ_{max}) and generation time (t_g).

Furthermore, the antimicrobial activity of *Lc. Lactis* CECT 4434 in supplemented cultivations with prebiotic fibers, in contrast with the strain *L. sakei* ATCC 15521, shows promise as food products may be created based on this symbiosis, which may be of commercial interest due to not having chemical preservatives, with the bacteriocin playing that role.

Acknowledgments

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ATTACHMENTS A – Statement for exemption of Ethics Committee approval.

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São Paulo, 05 de junho de 2017

DECLARAÇÃO DE DISPENSA DE AUTORIZAÇÃO DE COMITÊ DE ÉTICA

Eu, Sabrina da Silva Sabo (nº USP: 8439661), aluna do Departamento de Tecnologia Bioquímico-Farmacêutica na área de Tecnologia de Fermentações, sob orientação do Prof. Dr. Ricardo Pinheiro de Souza Oliveira, venho por meio desta, afirmar que o projeto de pesquisa intitulado como **Produção biotecnológica e aplicação de biomoléculas antimicrobianas por *Lactobacillus plantarum* cultivado em soro de leite**, dispensa autorização pelo Comitê de Ética, visto que não foram utilizados animais ou humanos para os experimentos propostos.

Sabrina da S. Sabo

Assinatura do aluno (a)

Ricardo P. S. Oliveira

Assinatura do orientador

ATTACHMENTS B – Student file

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
Documento sem validade oficial
FICHA DO ALUNO

9134 - 8439661/1 - Sabrina da Silva Sabo

Email:	sabrinasabo@usp.br
Data de Nascimento:	03/12/1985
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Local de Nascimento:	Estado de São Paulo
Nacionalidade:	Brasileira
Graduação:	Farmacêutica - Universidade São Francisco - São Paulo - Brasil - 2009

Curso:	Doutorado Direto
Programa:	Tecnologia Bioquímico-Farmacêutica
Área:	Tecnologia de Fermentações
Data de Matrícula:	05/02/2013
Início da Contagem de Prazo:	05/02/2013
Data Limite para o Depósito:	05/06/2017
Orientador:	Prof(a). Dr(a). Ricardo Pinheiro de Souza Oliveira - 05/02/2013 até o presente. Email: rpsolive@usp.br
Proficiência em Línguas:	Inglês, Aprovado em 05/02/2013
Prorrogação(ões):	120 dias Período de 05/02/2017 até 05/06/2017
Data de Aprovação no Exame de Qualificação:	Aprovado em 11/03/2015
Data do Depósito do Trabalho:	
Título do Trabalho:	
Data Máxima para Aprovação da Banca:	
Data de Aprovação da Banca:	
Data Máxima para Defesa:	
Data da Defesa:	
Resultado da Defesa:	
Histórico de Ocorrências:	Primeira Matrícula em 05/02/2013 Prorrogação em 08/12/2016

Aluno matriculado no Regimento da Pós-Graduação USP (Resolução nº 6542 em vigor a partir de 20/04/2013).
Última ocorrência: Matrícula de Acompanhamento em 06/02/2017
Impresso em: 18/05/2017 15:10:19

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
Documento sem validade oficial
FICHA DO ALUNO

9134 - 8439661/1 - Sabrina da Silva Sabo

Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação
FBT5773-7/1	Tópicos Especiais em Tecnologia Bioquímico-Farmacêutica	18/02/2013	28/04/2013	30	0	-	-	N	Pré-matrícula indeferida
FBA5898-7/1	Bacteriocinas e suas Aplicações em Alimentos	02/04/2013	22/04/2013	45	3	100	A	N	Concluída
FBT5729-6/4	Liofilização e suas Aplicações a Alimentos e à Medicamentos	03/04/2013	04/06/2013	90	6	100	A	N	Concluída
FBF5779-2/1	Preparo de Artigos Científicos na Área de Farmácia	16/08/2013	17/10/2013	90	0	-	-	N	Pré-matrícula indeferida
EDM5102-3/5	Preparação Pedagógica PAE (Faculdade de Educação - Universidade de São Paulo)	20/08/2013	30/09/2013	60	0	-	-	N	Pré-matrícula indeferida
FBT5776-4/9	Tópicos Especiais de Tecnologia Bioquímico-Farmacêutica II	07/10/2013	20/10/2013	30	2	100	A	N	Concluída
FBT5773-7/2	Tópicos Especiais em Tecnologia Bioquímico-Farmacêutica	07/10/2013	15/12/2013	30	2	100	A	N	Concluída
FBT5768-4/3	Princípios de Fermentação Contínua	07/02/2014	13/03/2014	75	5	100	A	N	Concluída
FBT5787-1/3	Aplicação Biotecnológica de Bactérias Láticas	04/03/2014	07/04/2014	45	3	100	A	N	Concluída
BTC5701-6/4	Metabolismo Microbiano (Curso Interunidades: Biotecnologia - Universidade de São Paulo)	07/03/2014	17/04/2014	90	0	-	-	N	Matrícula cancelada
BMM5729-1/3	Análise Sistêmica e Engenharia do Metabolismo Microbiano (Instituto de Ciências Biomédicas - Universidade de São Paulo)	23/04/2014	01/07/2014	120	8	100	A	N	Concluída
NEC5719-3/1	Preparação Pedagógica (Instituto de Psicologia - Universidade de São Paulo)	11/03/2015	29/04/2015	30	0	-	-	N	Matrícula cancelada
FBA5728-3/11	Aprimoramento Didático	14/04/2015	11/05/2015	60	0	-	-	N	Matrícula cancelada
BIP5700-7/1	Preparação Pedagógica em Biologia (Instituto de Biociências - Universidade de São Paulo)	04/05/2015	24/05/2015	45	0	-	-	N	Pré-matrícula indeferida

	Créditos mínimos exigidos		Créditos obtidos
	Para exame de qualificação	Para depósito de tese	
Disciplinas:	0	25	29
Estágios:			
Total:	0	25	29

Créditos Atribuídos à Tese: 167

Conceito a partir de 02/01/1997:

A - Excelente, com direito a crédito; B - Bom, com direito a crédito; C - Regular, com direito a crédito; R - Reprovado; T - Transferência.

Um(1) crédito equivale a 15 horas de atividade programada.

Última ocorrência: Matrícula de Acompanhamento em 06/02/2017

Impresso em: 18/05/2017 15:10:19

ATTACHMENTS C - Curriculum lattes

Sabrina da Silva Sabo

Endereço para acessar este CV: http://lattes.cnpq.br/0592792000732956
Última atualização do currículo em 19/05/2017

Possui graduação em Farmácia pela Universidade São Francisco (2009) e especialização em Microbiologia Ambiental/Industrial pela Sociedade Brasileira de Microbiologia (2012). Trabalhou durante 7 anos e 5 meses (06/2005-11/2012) no Laboratório Universitário de Análises Clínicas do Hospital Universitário São Francisco (Bragança Paulista - SP), dos quais 3 anos atuou como responsável técnica pelo setor de microbiologia. Atualmente está como aluna de doutorado-direto do departamento de Tecnologia Bioquímico-Farmacêutica na Faculdade de Ciências Farmacêutica da Universidade de São Paulo. Realizou Doutorado-Sanduíche no Departamento de Engenharia Química na Universidade de Vigo (Espanha) durante 12 meses. Amplo conhecimento em microbiologia clínica e industrial, sobretudo em processos biotecnológicos, com ênfase em fermentação, bem como, produção, purificação e aplicação de biomoléculas de interesse industrial. Também possui conhecimentos avançados na área de qualidade, uma vez que já participou e realizou auditorias para implantar ISO 9001 no laboratório de análises clínicas, o qual trabalhava. **(Texto informado pelo autor)**

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Nome em citações bibliográficas SABO, S. S.;DA SILVA SABO, SABRINA

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Título: Perfil de sensibilidade dos microrganismos causadores de sepse em Unidade de Terapia Intensiva do Hospital Universitário São Francisco-Bragança Paulista.
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Revisor de periódico

2014 - Atual	Periódico: African Journal of Food Science
2014 - Atual	Periódico: Chemical Engineering Transactions

Áreas de atuação

1.	Grande área: Ciências Biológicas / Área: Microbiologia.
2.	Grande área: Ciências da Saúde / Área: Farmácia.

Idiomas

Inglês	Compreende Bem, Fala Bem, Lê Bem, Escreve Bem.
Italiano	Compreende Pouco, Fala Pouco, Lê Pouco, Escreve Pouco.
Espanhol	Compreende Bem, Fala Bem, Lê Bem, Escreve Bem.

Produções

Produção bibliográfica

Artigos completos publicados em periódicos

Ordenar por

Ordem Cronológica

1. RODRÍGUEZ-PAZO, NOELIA ; **DA SILVA SABO, SABRINA** ; SALGADO-SEARA, JOSÉ MANUEL ; ARNI, SALEH AL ; DE SOUZA OLIVEIRA, RICARDO PINHEIRO ; DOMÍNGUEZ, JOSÉ MANUEL . Optimisation of cheese whey enzymatic hydrolysis and further continuous production of antimicrobial extracts by Lactobacillus plantarum CECT-221. Journal of Dairy Research (Print) JCR, v. 83, p. 402-411, 2016.

Citações: WEB OF SCIENCE℠ 1

2. **DA SILVA SABO, SABRINA**; CONVERTI, ATTILIO ; TODOROV, SVETOSLAV DIMITROV ; DOMÍNGUEZ, JOSÉ MANUEL ; DE SOUZA OLIVEIRA, RICARDO PINHEIRO . Effect of inulin on growth and bacteriocin production by *Lactobacillus plantarum* in stationary and shaken cultures. International Journal of Food Science & Technology (Print) JCR, v. 50, p. n/a-n/a, 2015.

Citações: WEB OF SCIENCE℠ 2

3. FARINHA, L. R. L. ; **SABO, S. S.** ; PORTO, M. C. ; SOUZA, E. C. ; OLIVEIRA, M. N. . Influence of Prebiotic Ingredients on the Growth Kinetics and Bacteriocin Production of Lactococcus lactis. Chemical Engineering Transactions, v. 43, p. 313-318, 2015.

Citações: SCOPUS 2

4. **DA SILVA SABO, SABRINA**; VITOLO, MICHELE ; GONZÁLEZ, JOSÉ MANUEL DOMÍNGUEZ ; OLIVEIRA, RICARDO PINHEIRO DE SOUZA . Overview of Lactobacillus plantarum as a promising bacteriocin producer among lactic acid bacteria. Food Research International JCR, v. 64, p. 527-536, 2014.

Citações: WEB OF SCIENCE℠ 8 | SCOPUS 13

Resumos publicados em anais de congressos

1. **SABO, S. S.**; PEREZ-RODRIGUEZ, N. ; GONZALEZ, J. M. D. ; OLIVEIRA, R. P. S. . Effect of hydrolysis of cheese whey proteins by Flavourzyme 1000 L on phenyllactic acid production by Lactobacillus plantarum. In: VI International Conference on Environmental, Industrial and Applied Microbiology, 2015, Barcelona. VI International Conference on Environmental, Industrial and Applied Microbiology - BioMicroWorld 2015, 2015. p. 147-147.

2. **SABO, S. S.**; LOPES, A. M. ; SANTOS-EBINUMA, V. C. ; PESSOA JUNIOR, A. ; GONZALEZ, J. M. D. ; OLIVEIRA, R. P. S. . Extraction of bacteriocin from the fermented broth of Lactobacillus plantarum ST16Pa using aqueous two-phase polymer systems. In: VI International Conference on Environmental, Industrial and Applied Microbiology, 2015, Barcelona. VI International Conference on Environmental, Industrial and Applied Microbiology - BioMicroWorld 2015.

3. **SABO, S. S.**; FARINHA, L. R. L. ; PORTO, M. C. ; GONZALEZ, J. M. D. ; OLIVEIRA, R. P. S. . Effect of different prebiotics on growth and bacteriocin production by Lactobacillus plantarum.. In: XXII Congresso Latino Americano de Microbiologia, 2014, Cartagena. Hechos Microbiológicos. Mendellín: Legis S.A., 2014. v. 5. p. 149-149.

4. ROSSO, B. U. ; PORTO, T. S. ; CONVETI, A. ; OLIVEIRA, R. P. S. ; **SABO, S. S.** ; PESSOA JUNIOR, A. . Clavulanate partition in two phase systems consisting of polyethylene glycol and polyacrylic acid in the presence of sodium chloride. In: XXII Congresso Latino Americano de Microbiologia, 2014, Cartagena. Hechos Microbiológicos. Mendellín: Legis S.A., 2014. v. 5. p. 153-153.

5. PORTO, M. C. ; FARINHA, L. R. L. ; **SABO, S. S.** ; OLIVEIRA, R. P. S. . Effect of prebiotics fructo-oligossacharides and polydextrose on growth cells of Pediococcus pentosaceus ATCC 43200 in semi-shyntetic broth.. In: XXII Congresso Latino Americano de Microbiologia, 2014, Cartagena. Hechos Microbiológicos. Mendellín: Legis S.A., 2014. v. 5. p. 151-151.

6. FARINHA, L. R. L. ; **SABO, S. S.** ; PORTO, M. C. ; OLIVEIRA, R. P. S. . Influencia de prebióticos en el crecimiento celular de Lactococcus lactis subsp. lactis CECT-221. In: XXII Congresso Latino Americano de Microbiologia, 2014, Cartagena. Hechos

Microbiológicos. Mendellín: Legis S.A., 2014. v. 5. p. 40-40.

7. FARINHA, L. R. L. ; **SABO, S. S.** ; PORTO, M. C. ; OLIVEIRA, R. P. S. . Celular Growth of Lactococcus lactis subsp.lactis CECT 4434 in synthetic broth. In: XIX Semana Farmacêutica de Ciência e Tecnologia da FCF/USP, 2014, São Paulo. Brazilian Journal of Pharmaceutical Science, 2014. v. 50. p. 31-31.
8. **SABO, S. S.**; OLIVEIRA, R. P. S. . BACTERIOCIN PRODUCTION BY Lactobacillus plantarum: DIFFERENCE BETWEEN AEROBIC AND ANAEROBIC CULTIVATION. In: 48ª Semana Universitária Paulista de Farmácia e Bioquímica, 2013, São Paulo. Brazilian Journal of Pharmaceutical Sciences. São Paulo: Brazilian Journal of Pharmaceutical Sciences, 2013. v. 49. p. 42-42.
9. BALCIUNAS, E. M. ; MARTINEZ, F. A. C. ; **SABO, S. S.** ; OLIVEIRA, R. P. S. . BACTERIOCIN PRODUCTION USING BIFIDOBACTERIUM LACTIS IN MILK WHEY. In: XXVII Congresso Latino Americano de Microbiologia, 2013, Natal. Anais do XXVII CBM, 2013.
10. MARTINEZ, F. A. C. ; BALCIUNAS, E. M. ; **DA SILVA SABO, SABRINA** ; LEE, R. ; OLIVEIRA, R. P. S. . OPTIMIZATION OF BACTERIOCIN PRODUCTION BY BIFIDOBACTERIUM LACTIS HN019 USING RESPONSE SURFACE METHODOLOGY. In: XXVII Congresso Latino Americano de Microbiologia, 2013, Natal. Anais do XXVII CBM, 2013.
11. **SABO, S. S.**; BALCIUNAS, E. M. ; MARTINEZ, F. A. C. ; OLIVEIRA, R. P. S. . CULTIVO E PRODUÇÃO DE BACTERIOCINA POR LACTOBACILLUS PLANTARUM: DIFERENÇA ENTRE ANAEROBIOSE E AEROBIOSE COM E SEM SUPLEMENTAÇÃO DE INULINA. In: XXVII Congresso Latino Americano de Microbiologia, 2013, Natal. Anais do XXVII CBM, 2013.

Apresentações de Trabalho

1. **SABO, S. S.**; SANTOS-EBINUMA, V. C. ; RANGEL-YUGUI, C. O. ; LOPES, A. M. ; OLIVEIRA, R. P. S. . Bacteriocin partitioning from the fermented broth of Lactobacillus plantarum ST16Pa in aqueous biphasic systems composed of polymers and cholinium-based salts. 2017. (Apresentação de Trabalho/Congresso).
2. **SABO, S. S.**; PÉREZ-PAZ, A. ; PÉREZ-RODRIGUES, N. ; VALLEJO, M. ; OLIVEIRA, R. P. S. ; GONZALEZ, J. M. D. . Screening for Bacteriocin-Like Substance by Patagonian Strains and their Ability yo Produce it Through Economic Conditions. 2016. (Apresentação de Trabalho/Simpósio).
3. **SABO, S. S.**; PEREZ-RODRIGUEZ, N. ; GONZALEZ, J. M. D. ; OLIVEIRA, R. P. S. . Effect of hydrolysis of cheese whey proteins by Flavourzyme 1000 L on phenyllactic acid production by Lactobacillus plantarum. 2015. (Apresentação de Trabalho/Congresso).
4. **SABO, S. S.**; LOPES, A. M. ; SANTOS-EBINUMA, V. C. ; PESSOA JUNIOR, A. ; GONZALEZ, J. M. D. ; OLIVEIRA, R. P. S. . Extraction of bacteriocin from the fermented broth of Lactobacillus plantarum ST16Pa using aqueous two-phase polymer systems. 2015. (Apresentação de Trabalho/Congresso).
5. **DA SILVA SABO, SABRINA**; AZEVEDO, P. S. ; OLIVEIRA, R. P. S. . Effect of different prebiotics on growth and bacteriocin production by Lactobacillus plantarum. 2015. (Apresentação de Trabalho/Simpósio).
6. **SABO, S. S.**; FARINHA, L. R. L. ; PORTO, M. C. ; GONZALEZ, J. M. D. ; OLIVEIRA, R. P. S. . Effect of different prebiotics on growth and bacteriocin production by Lactobacillus plantarum.. 2014. (Apresentação de Trabalho/Congresso).
7. PORTO, M. C. ; FARINHA, L. R. L. ; **SABO, S. S.** ; OLIVEIRA, R. P. S. . Effect of prebiotics fructo-oligossacharides and polydextrose on growth cells of Pediococcus pentosaceus ATCC 43200 in semi-shyntetic broth.. 2014. (Apresentação de Trabalho/Congresso).
8. FARINHA, L. R. L. ; **SABO, S. S.** ; PORTO, M. C. ; OLIVEIRA, R. P. S. . Influencia de prebióticos en el crecimiento celular de Lactococcus lactis subsp. lactis CECT-221. 2014. (Apresentação de Trabalho/Congresso).
9. ROSSO, B. U. ; PORTO, T. S. ; CONVETI, A. ; OLIVEIRA, R. P. S. ; **SABO, S. S.** ; PESSOA JUNIOR, A. . Clavulanate partition in two phase systems consisting of polyethylene glycol and polyacrylic acid in the presence of sodium chloride. 2014. (Apresentação de Trabalho/Congresso).
10. **SABO, S. S.**; BALCIUNAS, E. M. ; MARTINEZ, F. A. C. ; OLIVEIRA, R. P. S. . CULTIVO E PRODUÇÃO DE BACTERIOCINA POR LACTOBACILLUS PLANTARUM: DIFERENÇA ENTRE ANAEROBIOSE E AEROBIOSE COM E SEM SUPLEMENTAÇÃO DE INULINA. 2013. (Apresentação de Trabalho/Congresso).
11. BALCIUNAS, E. M. ; **SABO, S. S.** ; MARTINEZ, F. A. C. ; OLIVEIRA, R. P. S. . BACTERIOCIN PRODUCTION USING BIFIDOBACTERIUM LACTIS IN MILK WHEY. 2013. (Apresentação de Trabalho/Congresso).
12. MARTINEZ, F. A. C. ; **SABO, S. S.** ; BALCIUNAS, E. M. ; LEE, R. . OPTIMIZATION OF BACTERIOCIN PRODUCTION BY BIFIDOBACTERIUM LACTIS HN019 USING RESPONSE SURFACE METHODOLOGY. 2013. (Apresentação de Trabalho/Congresso).
13. **SABO, S. S.**; OLIVEIRA, R. P. S. . BACTERIOCIN PRODUCTION BY Lactobacillus plantarum: DIFFERENCE BETWEEN AEROBIC AND ANAEROBIC CULTIVATION. 2013. (Apresentação de Trabalho/Simpósio).

Bancas

Participação em bancas de comissões julgadoras

Outras participações

1. **SABO, S. S.**. Participou como membro do comitê para Avaliação dos Resumos submetidos para ao 22º Simpósio International de Iniciação Científica da USP. 2014. Faculdade de Ciências Farmacêuticas - USP.

Eventos

Participação em eventos, congressos, exposições e feiras

1. VI International Conference on Environmental, Industrial and Applied Microbiology. Extraction of bacteriocin from the fermented broth of Lactobacillus plantarum ST16Pa using aqueous two-phase polymer systems. 2015. (Congresso).
2. VI International Conference on Environmental, Industrial and Applied Microbiology. Effect of hydrolysis of cheese whey proteins by Flavourzyme 1000 L on phenyllactic acid production by Lactobacillus plantarum. 2015. (Congresso).
3. XXII Congresso Latino Americano de Microbiologia. Effect of different prebiotics on growth and bacteriocin production by Lactobacillus plantarum.. 2014. (Congresso).
4. 27º Congresso Brasileiro de Microbiologia. CULTIVO E PRODUÇÃO DE BACTERIOCINA POR LACTOBACILLUS PLANTARUM: DIFERENÇA ENTRE ANAEROBIOSE E AEROBIOSE COM E SEM SUPLEMENTAÇÃO DE INULINA. 2013. (Congresso).
5. 48ª Semana Universitária Paulista de Farmácia e Bioquímica.BACTERIOCIN PRODUCTION BY Lactobacillus plantarum: DIFFERENCE BETWEEN AEROBIC AND ANAEROBIC CULTIVATION. 2013. (Simpósio).
6. Workshop de Microbiologia. 2012. (Outra).
7. Capacitação de biossegurança. 2010. (Outra).
8. KPC, a superbactéria?. 2010. (Outra).
9. Resistência Bacteriana, do Teste de Sensibilidade à Biologia Molecular. 2010. (Seminário).
10. Treinamento "Espermograma". 2010. (Outra).
11. "I Simpósio de Análises Clínicas"."Doença de Chagas". 2009. (Simpósio).
12. Workshop de Microbiologia. 2009. (Outra).
13. Boas Práticas em Microbiologia Clínica. 2008. (Outra).
14. Coleta de Sangue a Vácuo. 2008. (Outra).
15. XXI Jornada Universitária Farmacêutica. 2008. (Outra).
16. Técnicas de Administração de Medicamentos Injetáveis. 2007. (Outra).
17. Terapia Nutricional no Paciente Grave. 2007. (Encontro).
18. XX Jornada Universitária Farmacêutica. 2007. (Outra).
19. Coleta de Sangue a Vácuo. 2006. (Outra).
20. Toxicologia de fármacos aplicada ao Laboratório de Análises Clínicas. 2006. (Outra).
21. XIX Jornada Universitária Farmacêutica. 2006. (Outra).
22. XVIII Jornanda Universitária Farmacêutica. 2005. (Outra).