UNIVERSITY OF SÃO PAULO
FACULTY OF PHARMACEUTICAL SCIENCES
Department of Biochemical and Pharmaceutical Technology

Optimization of the yield of bacteriocin-like substance (BLIS) produced by *Pediococcus pentosaceus* and its application as food bioconservative

Pamela Oliveira de Souza de Azevedo

Thesis to obtain the degree of Doctor

Advisor: Prof. Dr. Ricardo Pinheiro de Souza Oliveira

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RESUMO

AZEVEDO, P.O.S. **Otimização do rendimento de substância semelhante a bacteriocina (BLIS) produzido por *Pediococcus pentosaceus* e sua aplicação como bioconservante de alimentos.** 2018. 128 p. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2018.

Bacteriocinas são peptídeos produzidos por várias espécies de bactérias, especialmente bactérias ácido-láticas (BALs) e apresentam um amplo espectro de ação contra bactérias deteriorantes e patógenos de origem alimentar. Entretanto, quando estas bacteriocinas não foram completamente caracterizadas quanto a sequência de seus nucleotídeos e do seu gene correspondente, é recomendada a denominação de substância semelhante a bacteriocina (BLIS). Para aumentar a atividade antimicrobiana de bacteriocinas, a habilidade de BALs probióticas, como *Pediococcus pentosaceus*, em fermentar diferentes fontes de carbono e nitrogênio tem sido estudado. Para o desenvolvimento de um meio de cultura melhorado, fontes de carbono e nitrogênio devem ser consideradas como nutrientes responsáveis pelo crescimento celular e pela produção de bacteriocina. A melhor condição, após 48 h de cultivo, para o crescimento (3,420 g/L) e para a produção de BLIS por *P. pentosaceus* ATCC 43200 foi em meio de cultivo Man, Rogosa e Sharp (MRS) suplementado com 1,5% de peptona, pH inicial 6,0 e sob as seguintes condições de cultivo: anaerobiose, 30ºC e agitação de 200 rpm. Comparado ao controle (MRS sem suplementação), o crescimento de *Pediococcus* foi significativamente menor (1,995 g/L) assim, como também, reduziu significativamente o tempo de geração de 2,05 h (controle) para 1,28 h (MRS suplementado), uma redução de aproximadamente 62,5%. Além disso, a adição de peptona ao meio MRS promoveu redução de 4 h para o início da fase exponencial de *Pediococcus*. Quanto a atividade antimicrobiana de BLIS, a adição de fonte de nitrogênio ao meio MRS também foi bastante significativa. Através do método ágar difusão, BLIS apresentou halos de inibição entre 12,50 a 19,50 mm contra cepas de BALs (*Lactobacillus sakei* ATCC 15521, *Lactobacillus plantarum* CECT 221 e *Carnobacterium piscicola* CECT 4020). Contra cepas de *Listeria* (*Listeria innocua* NCTC 11288 e *Listeria seeligeri* NCTC 11289), a sua atividade inibitória foi melhor detectada em meio líquido, através da determinação da concentração mínima inibitória de 50%. BLIS sem diluição foi capaz de inibir 60 e 100% de *L. seeligeri* e *L. innocua*,

respectivamente, assim como, diluído 1x (v/v) em água foi capaz de inibir 100% o crescimento de ambas *Listeria*. BLIS também apresentou bons resultados como conservante de alimento quando aplicado em presunto contaminado artificialmente com *L. seeligeri* e armazenado a 4ºC a vácuo por 10 dias. BLIS foi capaz de manter baixa a multiplicação de *Listeria,* menor perda de peso das amostras, baixa peroxidação lipídica e bons parâmetros de cor durante o armazenamento das amostras. Os resultados demonstraram a importância de se otimizar meio de cultivo tanto para o aumento da massa microbiana como para a produção e melhoramento da atividade desta molécula antimicrobiana. Além disso, os resultados também sugerem a possível aplicação de BLIS como conservante natural de alimentos.

ABSTRACT

AZEVEDO, P.O.S. **Optimization of the yield of bacteriocin-like substance (BLIS) produced by *Pediococcus pentosaceus* and its application as food bioconservative.** 2018. 128 p. Thesis (Ph.D.) – Faculty of Pharmaceutical Sciences, University of São Paulo, 2018.

Bacteriocins are peptides produced by various species of bacteria, especially lactic acid bacteria (LABs), which exhibit a large spectrum of action against spoilage bacteria and foodborne pathogens. However, when this bacteriocin has not been completely characterized regarding its amino acid and the nucleotide sequences of the corresponding gene, the qualified term bacteriocin-like inhibitory substance (BLIS) is recommended. In order to increase the antimicrobial activity of bacteriocins, the ability of probiotics LABs, such as *Pediococcus pentosaceus*, to ferment different carbon and nitrogen sources has been studied. For the development of an improved culture medium, carbon and nitrogen sources must be considered as nutrients responsible for cell growth and bacteriocin production. The best condition, after 48 h of cultivation, for growth (3.420 g/L) and for BLIS production by *Pediococcus pentosaceus* ATCC 43200 was in Man, Rogosa and Sharp (MRS) culture medium supplemented with 1.5% peptone, initial pH 6.0 and under the following culture conditions: anaerobiosis, 30ºC and agitation of 200 rpm. Compared with control (MRS without supplement), the growth of *Pediococcus* was significantly lower (1.995 g/L) as well as it reduced significantly its generation time from 2.05 h (control) to 1.28 h (MRS supplemented), a reduction of approximately 62.5%. Moreover, addiction of peptone to MRS medium promoted reduction of 4 h to the *Pediococcus* exponential phase onset. Regarding BLIS antimicrobial activity, addition of nitrogen source to MRS medium was also quite significant. Through the agar diffusion method, BLIS showed inhibition halos between 12.50 and 19.50 mm against LABs strains (*Lactobacillus sakei* ATCC 15521, *Lactobacillus plantarum* CECT 221 and *Carnobacterium piscicola* CECT 4020). Against *Listeria* strains (*Listeria innocua* NCTC 111288 and *Listeria seeligeri* NCTC 11289), their antimicrobial activity was better detected in liquid medium assay, evaluating the minimal inhibitory concentration of 50%. BLIS was able to inhibit 60 and 100% of *L. seeligeri* and *L. innocua*, respectively, as well as, diluted 1x (v/v) in water was able to inhibit 100% growth of both *Listeria*. BLIS

showed also good results as food preservative when applied in ready-to-eat pork ham artificially contaminated with *L. seeligeri* in vacuum-package at 4^{o}C during shelf life of 10 days. BLIS was able to maintain low *Listeria* multiplication, lower samples weight loss, low lipid peroxidation and good color parameters during samples storage. Results demonstrated the importance of optimizing the culture medium to increase microbial mass, to produce and to improve the activity of this antimicrobial molecule. Moreover, results also suggest the possible application of BLIS as a natural food preservative.

CONTENTS

GENERAL INTRODUCTION

In food processing, all steps such as composition or quality of the products, their origin and how they have been handled, processed and stored have gained importance. The main reason for this is that consumers have become more aware about information on food quality. In this way, in recent years, the use of probiotic bacteria or their metabolites to prevent spoilage bacteria and foodborne pathogens growth, and to increase food shelf life, has become a growing and promising area for research in the food field with important food industrial application. Researches towards biotechnological production of natural antimicrobials, such as bacteriocin and/or bacteriocin-like substance (BLIS), are essential to provide the food industry natural food additives with potential application and with broad spectrum of action against spoilage bacteria and foodborne pathogens. *Pediococcus pentosaceus* ATCC 43200 is a *Pediococcus* specie with promising properties for use as probiotic culture. The antimicrobial activity of its molecule may be improved using optimal fermentations conditions. This antimicrobial substance has high activity against *Listeria monocytogenes* strains, thereby could become another important food additive to be use against foodborne pathogens and spoilage bacteria with quite promising future application as an alternative preservative in food industry. In this context, this thesis was divided into five chapters written in the form of articles. The chapter I comprise the influence of carbon sources (sucrose and inulin) to improve growth, lactate and bacteriocin production by *P. pentosaceus*. The chapter II demonstrated the importance of the agar-media in the evaluation of bacteriocin activity against the same test-microorganisms. In chapter III, it was studied the effect of pH and carbohydrate sources on growth of *P. pentosaceus* and the antimicrobial activity of its bacteriocin. In chapter IV, it was presented the optimal condition of fermentation by *P. pentosaceus* to produce BLIS with improved antimicrobial activity and, in chapter V, it was demonstrated the efficiency of BLIS as food bioconservative after its application in ready-to-eat pork ham artificially contaminated with *Listeria seeligeri* NCTC11289, showing the potential of this antimicrobial molecule in the preservation of food.

GENERAL AND SPECIFIC OBJECTIVES

The main objective of this thesis was the biotechnological production of bacteriocin-like substance (BLIS) produced by *Pediococcus pentosaceus* ATCC 43200 using glucose and peptone as carbon and nitrogen sources, respectively, and the evaluation of its application as food bioconservative. For this, the following specific objectives were established:

I. Optimize the growth of *Pediococcus pentosaceus* ATCC 43200 and its production of BLIS in bioreactor operated in discontinuous process using commercial culture medium (i.e. MRS) supplemented with different concentrations of nitrogen source (0.5, 1.0 and 1.5%), varying agitation (150 and 200 rpm) and aeration (microaerophilic and anaerobiosis);
II. Evaluate the antimicrobial activity of BLIS against food spoilage and foodborne pathogens in agar (i.e. agar diffusion method) and in liquid medium (i.e. minimal inhibitory concentration of 50% - MIC_{50});
III. Evaluate the efficiency of BLIS as food bioconservative after its application in ready-to-eat pork ham artificially contaminated with *Listeria seeligeri* NCTC 11289 and storage in vacuum-package at 4^{o}C during shelf life of 10 days.

CHAPTER I[1]

Positive influence of stimulating agents' sucrose and inulin on growth, lactate and bacteriocin production by *Pediococcus pentosaceus*

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ABSTRACT

Sucrose and inulin were investigated in this study as stimulating agents of bacteriocin production by *Pediococcus pentosaceus* ATCC 43200 when they were combined with glucose. When such a microbial strain was grown in glucose-based MRS medium, without additional supplements, it reached higher maximum cell concentration (2.68 g/L) and generation time (2.17 h), but lower specific growth rate (0.32 h^{-1}) than in the same medium supplemented with 1.0% of both ingredients (2.53 g/L, 1.60 h and 0.43 h^{-1}, respectively). Glucose replacement by sucrose or inulin almost completely suppressed growth, hence confirming that it is the preferred carbon source. Qualitatively, similar results were observed for lactate production, which was 59.8% higher in glucose-based medium. *Enterococcus* and *Listeria* strains were sensitive to bacteriocin, and both supplements in the glucose-based MRS medium improved the antimicrobial effect against these strains, and were also able to speed up *P. pentosaceus* in the exponential phase.

Keywords: *Pediococcus pentosaceus*, lactic acid bacteria, bacteriocin, pediocin, sucrose, inulin

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1. Introduction

Lactic acid bacteria (LAB) have a long history of safe use by man for food production and preservation and were first described in 1940 as starter cultures in fermented meat products (Jensen & Paddock, 1940). From then on, they have been widely used as starter cultures for fermentation in dairy, meet and other food industries (Mugula, Narvhus, & Sorhaug, 2002), and are nowadays recognized by the U.S. Food and Drug Administration as Generally Regarded as Safe (GRAS) microorganisms. LAB can be also used as cell factories in the production of an array of food additives and flavoring compounds (Ly et al., 2008; Kothari, Tyagi A, Patel, & Goyal, 2011).

Members of the *Pediococcus* genus, belonging to the group of LAB, are Gram-positive, homofermentative, non-motile, non-sporulating, facultative anaerobic cocci, usually arranged in tetrads (Zhang, Tong, & Dong, 2005). *Pediococcus pentosaceus* has been widely used in the fermentation of vegetables, meat and silage as well as in cheese production (Simpson & Taguchi, 1995). *P. pentosaceus* ATCC 43200 strain, also known as FBB61, was originally isolated in 1953 from fermented cucumber (Costilow, Coughlin, Robach, & Ragheb, 1956). The only pathway of sugars fermentation by homofermentative bacteria like *P. pentosaceus* is the Embden-Meyerhof-Parnas one, through which glucose cleavage leads to lactate formation. Their inhibitory activity was first observed by Etchells, Costilow, Anderson and Bell (1964) in pure culture fermentations of cucumbers and further investigated by Fleming, Etchells and Costilow (1975). Rueckert (1979) characterized the chemical structure of the inhibitory material, which was shown to be a non-dialysable protein localized in the semipermeable membrane, while Piva and Headon (1994) proved its bactericidal activity only a decade and a half later.

Bacteriocins are ribosomally-synthesized antimicrobial peptides produced by bacteria, which generally have a cationic and hydrophobic nature, a broad spectrum of action (Heng, Wescombe, Burton, Jack, & Tagg, 2007), thereby exhibiting antagonistic activity against several pathogens, and a lot of potential biotechnological applications (Bastos, & Ceotto, 2011; Cotter, Ross, & Hill, 2013). *Pediococcus* sp. bacteriocins are small, heat stable and non-lanthionine containing peptides belonging to the class II, which exhibit bactericidal activity against foodborne pathogens (Tagg, Dajani, & Wannamaker, 1976; Klaenhammer, 1988; Carminatt, Giraffa, & Bossi, 1989; Jiménez-Díaz, Rios-

Sanches, Desmazeaud, Ruiz-Barba, & Piard, 1993; Jack, Tagg, & Ray, 1995; Spellberg et al., 2008) such as *Listeria monocytogenes, Staphylococcus aureus* and *Escherichia coli*. Todorov and Dicks (2004) reported that bacteriocin production is strongly dependent on the type of nutrient source present in the culture medium, and that bacteriocin activity is not necessarily related to cell mass concentration or growth rate of the producer strain.

Inulin is a prebiotic, i.e., a carbohydrate that cannot be digested in the human gastrointestinal tract serving as a substrate for intestinal beneficial bacteria (probiotics), promoting their growth and improving their performance (Roberfroid, 2007). The stimulatory effect of this biopolymer on probiotics growth 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, 2011; Oliveira, Perego, Oliveira, & Converti, 2012).

Based on this background, the aim of the present work was to investigate the effect of sucrose and inulin supplementation in the glucose-based Man, Rogosa and Sharpe (MRS) medium on growth, sugar consumption, lactate and bacteriocin productions by *P. pentosaceus* ATCC 43200.

2. Material and Methods

2.1. Bacterial strains and growth conditions

P. pentosaceus ATCC 43200, the bacteriocin producer strain, was cultivated in 50 mL Man, Rogosa and Sharpe (MRS) medium (Difco Laboratories, Detroit, MI, USA) at pH 6.5 during 16 h at 37ºC with aeration at 100 rpm. *Enterococcus* sp. (strains EN101, EN104, EN711), *Listeria innocua* (strains LI2052, LI2865) and *Escherichia coli* ATCC 25922 were used as indicator strains. *Enterococcus* and *Listeria* strains were grown in Brain Heart Infusion (BHI) medium. *E. coli* was grown in Tryptic Soy Broth (TSB) (Difco). One mL of each cryopreserved indicator strain was grown in 5.0 mL medium for 16 h at 37ºC without agitation. The cultures were then diluted with sterile deionized water to give an optical density at 600 nm of 0.6, corresponding to 2.6×10^{10} CFU/mL for *Enterococcus* strains, 1×10^{10} CFU/mL for LI 2052, 2.7×10^{10} CFU/mL for LI2865 and 2×10^{10} CFU/mL for *E. coli*.

2.2. Culture medium preparation

The MRS medium pH 6.5 that contains 20 g/L glucose (G-MRS) was prepared according to the manufacturer and supplemented with either 1.0% (w/v) inulin (Orafti®GR, Orafti Active Food Ingredients, Oreye, Belgium) (GI-MRS), 1.0% (w/v) sucrose (GS-MRS), 1.0% (w/v) inulin and sucrose (GSI-MRS). Non-containing glucose media were prepared as the G-MRS medium, but replacing 20 g/L glucose by sucrose (S-MRS) or inulin (I-MRS) in the same proportion. All culture media were sterilized at 121ºC for 15 min.

2.3. Inoculum preparation and cultivation

To prepare the *P. pentosaceus* pre-inoculum, 1.0 mL of cryopreserved culture was inoculated into 100 mL Erlenmeyer flasks containing 50 mL of MRS medium pH 6.5. Flasks were placed on a rotary shaker at 37ºC with aeration at 100 rpm for 16 h. The growth curve was followed to give an optical density at 600 nm of 0.8-0.9, corresponding to 10^7 CFU/mL. Ten mL of the pre-inoculum were transferred into 250 mL Erlenmeyer flasks containing 100 mL of MRS medium pH 6.5 and incubated in a rotary shaker at 30ºC with aeration at 100 rpm. Aliquots were taken every 2 h during the first 12 h of the cultivation, and thereafter at 24 h and 48 h in triplicate.

2.4. Bacteriocin activity determination

To determine bacteriocin activity, aliquots were centrifuged at 4,470×*g* at 4ºC for 15 min. The pH of cell-free supernatants (CFSs) was adjusted to 6.0-6.5 by addition of 1.0 N NaOH, heated to 70ºC for 25 min to inactivate proteases, and sterilized by filtration through filters with 0.45 µm pore diameter (Millipore, Billerica, MA, USA).

The agar well diffusion assay was performed using 200 µL of each indicator strain suspension (OD 0.6) added to 20.0 mL of medium supplemented with 1.0 % (w/v) agar and poured into Petri dishes. Fifty µL of each denatured supernatant was added into each well. All plates were incubated for 16-18 h at 37ºC in duplicate. The antagonistic activity in arbitrary unit/mL (AU/mL) was calculated (Bhaskar, Sudeepa, Rashmi, & Tamil Selvi, 2007) as a measure of bacteriocin production.

$$AU/mL = \frac{\text{Diameter of the zone of clearence (mm) x 1000}}{\text{Volume taken in the well (µL)}}$$

2.5. Hydrogen peroxide production

To exclude any possible inhibitory effect associated to the release of hydrogen peroxide, which would interfere with bacteriocin activity, a double-agar plate assay was performed. One mL of *P. pentosaceus* cryopreserved culture was cultivated in 50 mL of MRS medium for 18 h at 30ºC with aeration at 100 rpm. Fifteen mL of MRS supplemented with 1.5% (w/v) agar were poured into Petri dishes. One hundred µL of *P. pentosaceus* suspension (OD 0.8) were spread onto the plates and incubated for 48 h at 37ºC under either aerobic condition (with aeration at 100 rpm) or anaerobic condition (jar BBL GasPack®System, BioQuest, Cockeysville, MD, USA). BHI medium supplemented with 1.0% (w/v) agar and 10.0 mL of EN101 suspension (OD 0.6) were poured into the above *Pediococcus*-containing Petri dishes. The plates were again incubated under aerobic or anaerobic atmosphere for 24 h at 37ºC.

2.6. Analytical procedures

Cell mass concentration of *P. pentosaceus* during the cultivation was determined by optical density at 600 nm using a calibration curve (R^2 = 0.997) of OD versus dry weight (DW) and expressed in g_{DW}/L. The progressive acidification of culture medium during the cultivation was followed through a pHmeter, model 400M1 (Quimis, Diadema, SP, Brazil). The concentrations of glucose and lactate were determined by means of a High Performance Liquid Chromatograph (HPLC), model LC-20A Prominence (Shimadzu, Kyoto, Japan), equipped with two LC-20AD pumps, a DGU-20A degasser unit, a SIL-20ACHT self-injector, a CTO-20AC column oven, a RI-210 refractive index detector (Shodex, Kawasaki, Kanagawa, Japan) and a HPX-87H column (300 x 7.8 mm) (Aminex, Bio-Rad, CA, USA). Analyses were carried out at room temperature using 75:25 % acetonitrile:ultrapure water as mobile phase at a flow rate of 0.9 mL/min. High purity glucose and lactate (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. All samples were assessed in triplicate.

2.7. Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's test by Statistica Software 12 (Tulsa, OK, USA) were performed and a significant difference was defined as a p value < 0.05.

3. Results and Discussion

3.1. Growth kinetics, acidification profiles and lactate production

As shown in Table 1, *P. pentosaceus* was able to grow in glucose-containing medium (G-MRS) or in G-MRS supplemented with sucrose (GS-MRS), with inulin (GI-MRS) or with both supplements (GSI-MRS). Taking into account that all the above media had the same glucose content and that those containing only sucrose (S-MRS) or only inulin (I-MRS) hardly allowed the growth, indicated that these supplements were not efficient as the only carbon source to the *P. pentosaceus* metabolism, confirming that glucose is the preferred carbon source for this strain as well as observed to all LAB (Saminathan, Sieo, Kalavathy, Abdullah, & Ho, 2011). Furthermore, the slight delay in the early exponential growth phase (about 6 h) compared with those media containing glucose (4 h) can only be ascribed to the additional presence of glucose (data not shown). Under all the tested conditions, *P. pentosaceus* achieved the stationary growth phase after 12 h and stopped growing after 24 h.

Some studies have demonstrated the potential use of *Pediococcus* sp. as probiotic candidates (Casey et al., 2007; Carey, Kostrzynska, Ojha, & Thompson, 2008), but their symbiotic effects together with prebiotics were poorly investigated. Carbohydrate preference of probiotics varies widely even within a single species, and also depends on the eventual presence of different prebiotics (Mei, Carey, Tosh, & Kostrzynska, 2011), among which oligosaccharides are preferred over monosaccharides in most cases (Amaretti et al., 2007). The ability of some strains to metabolize prebiotics is strain-specific and may be associated with the presence of specific plasmids (Mei, Carey, Tosh, & Kostrzynska, 2011).

Table 1. Kinetic parameters in different media fermented by *P. pentosaceus* ATCC 43200 performed up to 48 h

	Culture conditions					
	G-MRS	GS-MRS	GI-MRS	S-MRS	I-MRS	GSI-MRS
Cell concentration (g/L)	2.68 ± 1.10^{d}	1.76 ± 0.80^{b}	1.76 ± 0.80^{b}	0.11 ± 0.10^{a}	0.13 ± 0.10^{a}	2.53 ± 1.10^{c}
μ_{max} (h^{-1})	0.32 ± 0.00^{b}	0.33 ± 0.00^{c}	0.32 ± 0.00^{b}	0.12 ± 0.00^{a}	0.12 ± 0.00^{c}	0.43 ± 0.00^{d}
t_g (h)	2.17 ± 0.02^{a}	2.12 ± 0.04^{a}	2.13 ± 0.02^{a}	5.70 ± 0.03^{c}	5.80 ± 0.02^{d}	1.60 ± 0.05^{b}

Legend: G-MRS = glucose-based MRS medium, GS-MRS = glucose + sucrose-based MRS medium, GI-MRS = glucose + inulin-based MRS medium, S-MRS = sucrose-based MRS medium, I-MRS = inulin-based MRS medium, GSI-MRS = glucose + sucrose + inulin-based MRS medium. Mean values (n = 3) ± standard deviations. Different letters in the same line mean that values significantly differ among them ($p < 0.05$).

In G-MRS medium supplemented with both 1.0 % sucrose and inulin (GSI-MRS), maximum cell concentration after 48 h of cultivation (X_{max} = 2.53 g/L dry weight) and generation time in the exponential phase (T_g = 1.60 h) were lower than those obtained in the same medium without any supplement (G-MRS) (X_{max} = 2.68 g/L dry weight, T_g = 2.17 h), and maximum specific growth rate was higher (μ_{max} = 0.43 h^{-1} instead of 0.32 h^{-1}), thereby demonstrating that the combination of glucose, sucrose and inulin was able to speed up *P. pentosaceus* growth. In G-MRS medium supplemented with only 1.0% sucrose (GS-MRS) or 1.0% inulin (GI-MRS), cell concentration after the same time (X_{max} = 1.76 g/L dry weight) was the same and, as expected, maximum specific growth rate (μ_{max} = 0.33 h^{1} and 0.32 h^{-1}, respectively) and generation time (T_g = 2.12 h and 2.18 h, respectively) were practically coincident (Table 1).

It should be noted that, consistently with the fact that glucose is the preferred carbon source to this microorganism, its maximum specific growth rate (μ_{max} = 0.32 h^{-1}) and generation time (T_g = 2.17 h) in the G-MRS medium were coincident with those obtained in the GS-MRS and GI-MRS broths (Table 1). Pranckuté, Kaunietis, Kuisiené, and Citavicius (2014), who studied the influence of carbon sources (glucose and inulin) on *Lactobacillus* sp. and *Lactococcus* sp. growth, observed the fastest growth of *Lactococcus lactis* subs. *lactis*, *Lactobacillus sakei* and *Lactobacillus acidophilus* only in the presence of glucose as a carbon source, whereas inulin assimilation was completely ineffective with all the bacteria and strongly delayed the occurrence of the stationary growth phase.

To shed light on the above apparently surprising effect of inulin, one can give a look at the time behavior of yields of biomass ($Y_{X/S}$) and lactate ($Y_{P/S}$) on consumed glucose (Table 2). First, it is noteworthy that these yields are complementary one to another, in that any increase in the former implied a decrease in the latter. Based on this observation, one can see that *P. pentosaceus*, irrespectively of the presence or not of supplements in the glucose-based MRS medium, always exhibited a 4 h long lag phase, during which it consumed glucose by homolactic fermentation to lactate exclusively for maintenance purposes, without any growth.

During the exponential phase of growth, between 4 and 10 h, this microorganism utilized an increasing fraction of substrate to grow, reaching a maximum value of $Y_{X/S}$ and a minimum value of $Y_{P/S}$. Finally, during the stationary phase (since 10 h onwards), $Y_{P/S}$ increased at the expense of $Y_{X/S}$, and product formation became progressively more important compared with growth. Although these trends were qualitatively similar in the

presence and the absence of 1.0% of both sucrose and inulin, these supplements favored cell growth at the expense of lactate production during the whole stationary phase as well as the late exponential growth phase (data not shown), thus being responsible for the above-mentioned highest μ_{max} value.

Resuming, the early-supposed growth-stimulating effect of fructose moiety of sucrose (and to less extent of inulin), in the simultaneous presence of glucose, should be ascribed to some ability of *P. pentosaceus* to uptake fructose under conditions of incipient glucose depletion. This explanation appears to be consistent not only with the well-known prebiotic effect of fructose-based polysaccharides (inulin) (Bruno, Lankaputhra, & Shah, 2002; Akalm, Fenderya, & Akbulut, 2004; Oliveira, Perego, Converti, & Oliveira, 2009), oligosaccharides (Euler, Mitchell, Kline, & Pickering, 2005) or even sucrose (Mundt, Beattie, & Wieland, 1969; Suzuki, Ozaki, & Yamashita, 2004; Suzuki, Sami, Iijima, Ozaki, & Yamashita, 2006; Calmin, Lefort, & Belbahri, 2008), but only with some consumption of sucrose as a co-substrate, which, according to simple material balances, seemed to start just after the end of the exponential phase (about 14% after 10 h) and to become progressively more significant during the stationary one (about 23% after 48 h).

As shown in Table 2, lactate production (10.40 g/L) was the highest and almost coincident in G-MRS, GS-MRS and GI-MRS media, significantly lower (6.50 g/L) in GSI-MRS medium and almost absent either in S-MRS or I-MRS broth (0.48 g/L) ($p < 0.05$), confirming the unsuitability of sucrose and inulin as the only carbon source. This acidification pattern of *P. pentosaceus* is expected to contribute, if ingested as a probiotic, to decrease the colonic pH and then inhibit non-acid-tolerant bacteria. In addition, high concentration of lactate could prevent the proliferation of food-borne pathogens and spoilage organisms (Adams, & Hall, 1988; Holyoak et al., 1996; Cintas et al., 2001) via a pH decrease.

Table 2. Fermentative parameters in different media fermented by *P. pentosaceus* ATCC 43200 performed up to 48 h

	Fermentative parameters					
Culture media	Glucose (g/L)	Lactate (g/L)	$Y_{X/S}$ (g/L)	$Y_{P/S}$ (g/g)	Qp (g/L.h)	η (%)
G-MRS	2.01 ± 0.01^{c}	10.40 ± 0.13^{c}	0.15 ± 0.00^{b}	0.81 ± 0.01^{a}	0.21 ± 0.00^{c}	0.90 ± 0.01^{a}
GS-MRS	2.01 ± 0.01^{c}	10.20 ± 0.07^{bc}	0.10 ± 0.00^{a}	0.80 ± 0.00^{a}	0.21 ± 0.00^{bc}	0.89 ± 0.00^{a}
GI-MRS	2.01 ± 0.00^{c}	10.00 ± 0.04^{b}	0.10 ± 0.00^{a}	0.80 ± 0.01^{a}	0.21 ± 0.00^{a}	0.89 ± 0.02^{a}
S-MRS	1.18 ± 0.01^{b}	0.47 ± 0.01^{a}	0.09 ± 0.01^{a}	0.02 ± 0.01^{b}	0.01 ± 0.00^{a}	0.02 ± 0.01^{b}
I-MRS	0.57 ± 0.01^{a}	0.48 ± 0.03^{a}	0.24 ± 0.01^{c}	0.03 ± 0.00^{b}	0.01 ± 0.00^{a}	0.03 ± 0.00^{b}
GSI-MRS	12.82 ± 0.01^{d}	6.50 ± 0.01^{d}	0.35 ± 0.00^{d}	0.56 ± 0.00^{c}	0.14 ± 0.00^{d}	0.06 ± 0.00^{c}

Legend: G-MRS = glucose-based MRS medium, GS-MRS = glucose + sucrose-based MRS medium, GI-MRS = glucose + inulin-based MRS medium, S-MRS = sucrose-based MRS medium, I-MRS = inulin-based MRS medium, GSI-MRS = glucose + sucrose + inulin-based MRS medium, $Y_{X/S}$ = coefficient conversion of substrate (glucose) into biomass, $Y_{P/S}$ = coefficient conversion of substrate (glucose) into product (lactate), Qp = volumetric productivity of lactate, η = lactate yield. Mean values ($n = 3$) ± standard deviations. Different letters in the same column mean that values significantly differ among them ($p < 0.05$).

3.2. Glucose consumption

In G-MRS medium no less than 89.9% of starting glucose (20.0 g/L) was consumed after 48 h of cultivation (Table 2), compared with only 35.9% in the GSI-MRS one ($p < 0.05$). Whereas the percentage of glucose uptake was the same in G-MRS, GS-MRS and GI-MRS media (90%). As mentioned above, *P. pentosaceus* was unable to uptake sucrose or inulin as the only carbon source from S-MRS or I-MRS media, respectively. According to these results (not shown), it is evident that glucose must be present as the main carbon source for optimum *P. pentosaceus* growth and lactate production, and that sucrose and inulin are not effectively involved in these metabolic activities.

3.3. Agar well diffusion assay

The antimicrobial activities of cell-free supernatants (CFSs) were assessed in the exponential (8 h) and stationary (24 h) phases taken *Enterococcus* and *L. innocuas* strains as examples of Gram-positive bacteria and *E. coli* as Gram-negative. *Enterococcus* and *Listeria* strains showed to be sensitive to *P. pentosaceus* CFSs however, *Listeria* strains were the most sensitive, showing more than 300 AU/mL. There was no antimicrobial effect against *E. coli* (Table 3).

It was evident the importance of glucose presence in the medium but, for some strains, such as EN101, LI2052 and LI2865 (Table 3), the additional presence of sucrose and inulin improved bacteriocin antimicrobial activity. However, it is important to highlight that sucrose and inulin demonstrated to be not effective to the metabolism of this *P. pentosaceus* strain as regard the bacteriocin production when they are the only carbon sources present in the medium.

Pranckuté, Kaunietis, Kuisiené and Citavicius (2014), in their effort to investigate the influence of different carbon sources (glucose, inulin and palatinose, a reducing sugar composed of glucose and fructose moieties) on bacteriocin production by *Lactobacillus* sp. and *Lactococcus* sp., observed that a) *Lactococcus* species grew faster with better bacteriocin activity than the *Lactobacillus* ones in glucose-based medium, b) bacteriocin activity was not related to cell density and c) the effect of these oligosaccharides was strain-specific. *L. lactis* strains did in fact assimilate well palatinose but ineffectively inulin, except *L. lactis* DSM20729 that equally poorly assimilated both, producing only

20 AU/mL of bacteriocin after 6 h of growth, while in a glucose-based medium its activity was twice as higher. On the other hand, *L. sakei* and *L. acidophilus* were ineffective in the uptake of both components. The results obtained by those authors with *Lactococcus* sp. are somehow similar to those obtained in the present work, in that, inulin and sucrose were ineffective when used as the only carbon source, but behaved as stimulating agents when added at low concentration to a glucose medium.

As demonstrated in Table 3, there were statistical differences in the antimicrobial activity, expressed in AU/mL, of CFSs when the samples were collected in the exponential (8 h) and stationary (24 h) phases, demonstrating that this bacteriocin production is correlated with *P. pentosaceus* growth. The correlation of bacteriocin production with bacteria growth, implying that the volumetric bacteriocin production is dependent on the total biomass formation, was also observed by others authors (De Vuyst, & Vandamme, 1992; Moortvedt-Abildgaard et al., 1995; De Vuyst, Callewaert, & Crabbé, 1996). According to Callewaert and De Vuyst (2000), after reaching a maximal bacteriocin activity in the fermentation medium during the active growth phase a drastic decrease in soluble bacteriocin activity occurs, in order to proteolytic inactivation (Joerger, & Klaenhammer, 1986; De Vuyst, & Vandamme, 1992), protein aggregation (De Vuyst, Callewaert, & Crabbé, 1996; De Vuyst, Callewaert, & Pot, 1996), and adsorption of the bacteriocin molecules to the cell surface of the producer cells (De Vuyst, Callewaert, & Crabbé, 1996; Yang, Johnson, & Ray, 1992; Parente, & Ricciardi, 1994; Parente, Brienza, Ricciardi, & Addario, 1997).

Table 3. Quantification of cell-free supernatant of *P. pentosaceus* ATCC 43200 cultivated in different media and collected at 8 and 24 h

		Antimicrobial activity (AU/mL) at 8 and 24 h					
Time (h)	**Indicator strains**	G-MRS	GS-MRS	GI-MRS	S-MRS	I-MRS	GSI-MRS
8	*Enterococcus* sp. 101	120.25 ± 0.35^{b}	144.00 ± 1.41^{e}	171.00 ± 1.41^{f}	0.00	0.00	135.00 ± 1.41^{d}
24		100.10 ± 0.14^{a}	103.00 ± 1.41^{a}	101.50 ± 2.12^{a}	0.00	0.00	127.50 ± 0.71^{c}
8	*Enterococcus* sp. 104	152.35 ± 0.49^{d}	143.40 ± 0.57^{c}	170.50 ± 0.71^{e}	0.00	0.00	113.00 ± 1.41^{b}
24		113.85 ± 0.21^{b}	102.40 ± 0.57^{a}	100.50 ± 0.07^{a}	0.00	0.00	101.50 ± 2.12^{a}
8	*Enterococcus* sp. 711	178.25 ± 0.35^{f}	131.00 ± 1.41^{c}	166.25 ± 0.35^{d}	0.00	0.00	110.50 ± 0.71^{b}
24		170.50 ± 0.71^{e}	102.00 ± 1.41^{a}	101.00 ± 1.41^{a}	0.00	0.00	100.35 ± 0.49^{a}
8	*Listeria innocua* 2052	327.20 ± 0.28^{e}	306.20 ± 0.28^{c}	300.50 ± 0.71^{a}	0.00	0.00	333.10 ± 0.14^{g}
24		330.00 ± 0.00^{f}	300.00 ± 0.00^{a}	260.50 ± 0.71^{b}	0.00	0.00	321.00 ± 1.41^{d}
8	*Listeria innocua* 2865	330.10 ± 0.28^{g}	300.20 ± 0.28^{e}	339.00 ± 1.41^{a}	0.00	0.00	340.50 ± 0.71^{a}
24		310.00 ± 0.00^{f}	260.10 ± 0.00^{b}	287.20 ± 0.28^{d}	0.00	0.00	275.50 ± 0.70^{c}
8	*Escherichia coli* ATCC 25922	0.00	0.00	0.00	0.00	0.00	0.00
24		0.00	0.00	0.00	0.00	0.00	0.00

Legend: G-MRS = glucose-based MRS medium, GS-MRS = glucose + sucrose-based MRS medium, GI-MRS = glucose + inulin-based MRS medium, S-MRS = sucrose-based MRS medium, I-MRS = inulin-based MRS medium, GSI-MRS = glucose + sucrose + inulin-based MRS medium. Mean values (n = 3) ± standard deviations. Different letters in the same column mean that values significantly differ among them ($p < 0.05$).

3.4. Hydrogen peroxide production

To establish whether the antimicrobial activity of CFSs from *P. pentosaceus* cultivation was not related to the possible release of H_2O_2 (hydrogen peroxide) under aerobic conditions, this strain was also grown under anaerobic conditions, and the results in terms of colonies grown on Petri plates are illustrated in Figure 1. One can see that the strain selected for this work exerted antimicrobial activity under both conditions against EN101 selected as a target microorganism, thus proving that its antimicrobial activity was not related to any H_2O_2 production.

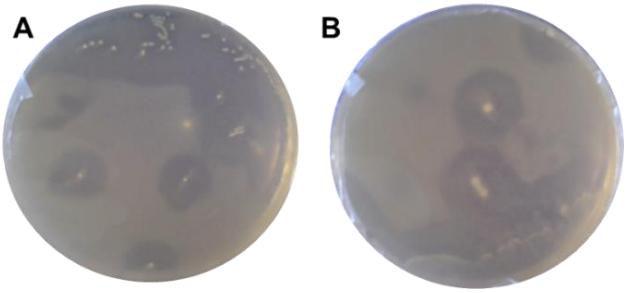

Figure 1. Micrographs of plates where *P. pentosaceus* ATCC 43200 and subsequently EN101 were grown for 48 and 24 h, respectively, under aerobic (A) or anaerobic (B) conditions, according to the double-agar plate method.

4. Conclusion

The results of this work allowed us to conclude that glucose is the carbohydrate preferentially metabolized as a carbon and energy source by *P. pentosaceus* ATCC 43200. Nevertheless, when the aim is bacteriocin production, glucose must be present regardless the combination with other carbon source such as inulin or even sucrose, to improve bacteriocin activity. Even without purification, the bacteriocin produced by this strain have shown to exert an important antimicrobial activity against microorganisms belonging to the *Listeria* genus suggesting the possibility of the use of this bacteriocin in future industrial applications as an alternative of antimicrobial food additive.

CHAPTER II[1]

Importance of the agar-media in the evaluation of bacteriocin activity against the same test-microorganisms

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ABSTRACT

Bacteriocins are peptides produced by various species of bacteria, especially lactic acid bacteria, which exhibit a large spectrum of action against spoilage bacteria and foodborne pathogens. Successful application of techniques for quantitative or qualitative bacteriocin determination relies not only on the sensitivity of the test-microorganisms, but also on the agar-medium employed. Cell free supernatants are routinely used to preliminary screen antimicrobial activity of bacteria by agar well diffusion method, but the supernatant may also include other molecules (such as medium components and/or intracellular compounds) accidentally released during cell free supernatant preparation, which may interfere with the assay. Reproducibility of bacteriocin activity against the same test-microorganisms is an important factor to be considered. Unfortunately, no specific information about standardization bioassays to determine bacteriocin activity is available in the literature. In this work, growth inhibition using agar well diffusion assays were carried out on different agar-media showing a strong dependence on the agar-medium used, indicating that the inhibitory effects could also depend on the diffusion of exudates that are included in the cell free supernatant. The results presented in this communication show that selection of the agar-medium is crucial for the bioassay response.

Keywords: Bacteriocin, Growth inhibition, Solid medium, Agar well diffusion assay

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1. Introduction

Bacteriocins are small and cationic antimicrobial peptides, ribosomally synthesized by lactic acid bacteria (LAB) (Singh, & Ghosh, 2012), with antilisterial activity (Papagianni, & Sergelidis, 2015) and many industrial applications (Papagianni, 2003). The industrial importance of LAB is further evinced by their generally recognized as safe (GRAS) status (Fan, & Song, 2013). In addition, lactic acid and other metabolic products produced by LAB contribute to the organoleptic and textural profile, as well as shelf life of the foods (Ross, Morgan, & Hill, 2002), especially the ones produced from fermented milk cultures concerning for the control of foodborne pathogens (Porto, Kuniyoshi, Azevedo, Vitolo, Oliveira, 2017).

Several pediocins, the bacteriocins produced by *Pediococcus pentosaceus* spp., have been characterized (Papagianni, & Anastasiadou, 2009) and they are amongst the most promising bacteriocin in the industry (Turcotte, Lacroix, Kheard, Grignon, & Fliss, 2004) as food preservative (Bharti et al., 2015). They have structural similarities, but different spectrum of antimicrobial activity (Papagianni, & Sergelidis, 2015). Pediocins exhibit important technological properties such as thermostability and retaining of activity at a wide pH range with bactericidal action especially against Gram-positive food spoilage and pathogenic bacteria; these features make pediocins an important class of biopreservatives (Papagianni, & Anastasiadou, 2009).

P. pentosaceus ATCC 43200, also known as FBB61, was isolated in 1953 from cucumber fermentation and produced pediocin A (Fleming et al., 1975), which was shown to belong to the class III bacteriocins (Klaenhammer, 1993), with molecular weight of 80 kDa and a broad range of activity against Gram-positive bacteria (Piva, & Headon, 1994).

A major difficulty in antimicrobial peptide research and applications is their identification and quantification using bioassays (Choyam, Lokesh, Bheemakere, & Kammara, 2015). Agar diffusion assay, that produces halos where growth is inhibited, is undoubtedly the most commonly used method to determine bacteriocin activity (Bouksaim, Lacroix, Audet, & Simard, 2000). However, this methodology is dependent on the bacteriocin diffusing through the agar, it is time-consuming and also relies on human interpretation when zones of inhibition are unclear or not perfectly circular (Papagianni, Avramidis, Filioussis, Dasiou, & Ambrosiadis, 2006). The possibility of

unspecific reaction between the active substance present in the tested culture and the agar-medium should also be considered.

The aim of this work was to illustrate just how variable seemingly zones of inhibition can be when bacteriocin activity against *Enterococcus* spp., *Listeria* spp. and *Escherichia coli* was assessed using agar diffusion test performed in different types of agar-media.

2. Material and Methods

2.1. Bacterial strains and growth conditions

P. pentosaceus ATCC 43200 (OD_{600nm} = 0.8-0.9) (Halo VIS-10, Dynamica, Australian) was grown at 30ºC with agitation at 100 rpm (TE-420, Tecnal, Piracicaba, SP, Brazil), in 100 mL of Man, Rogosa and Sharpe (MRS) medium pH 6.5 (Difco Laboratories, Detroit, MI, USA), which contains 2.0% (w/v) as main C-source (G-MRS). It was supplemented with either 1.0% (w/v) inulin (Orafti®GR, Orafti Active Food Ingredients, Oreye, Belgium) (GI-MRS), 1.0% (w/v) sucrose (GS-MRS), 1.0% (w/v) inulin and 1.0% (w/v) sucrose (GSI-MRS); alternatively, 2.0% (w/v) inulin (I-MRS) and 2.0% (w/v) sucrose (S-MRS) were used as the sole carbon source. Aliquots were taken every 2 h during the first 12 h of the cultivation, and thereafter at 24 h and 48 h.

Enterococcus spp. (strains Ent101, Ent104 and Ent711), *Listeria innocua* (strains Li2052 and Li2865) were grown in Brain Heart Infusion (BHI) medium (Difco). *Escherichia coli* ATCC 25922 was grown in Tryptic Soy (TSB) Broth (Difco). One mL of each cryopreserved bacterium was grown in 5.0 mL medium for 16 h at 37ºC without agitation (TE-310, Tecnal, Piracicaba, SP, Brazil). The cultures were then diluted with sterile deionized water to give an optical density at 600 nm of 0.3.

2.2. Bacteriocin activity determination

P. pentosaceus cells were collected by centrifugation (4.470 x *g* at 4ºC for 15 mins) and the cell-free supernatant was adjusted to pH 6.0-6.5 by the addition of 1.0 N NaOH, heated to 70ºC for 25 min to inactivate proteases and filtered (0.45 µm pore

diameter filter, Millipore, Billerica, MA, USA). The agar well diffusion assay was performed using 200 µL of each indicator strain suspension (OD = 0.3) (Halo VIS-10, Dynamica, Australian) added to 20.0 mL of medium supplemented with 1.0% (w/v) agar and poured into Petri dishes. Fifty µL of each denatured supernatant was added into each well. All plates were incubated for 16-18 h at 37ºC (TE-310, Tecnal, Piracicaba, SP, Brazil) in duplicate. The agar-media evaluated were BHI and Mueller Hinton to *Enterococcus* and *Listeria* strains and TSB and MacConkey to *E. coli*.

2.3. Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's test by Microsoft Excel (Redmond, WA, USA) were performed and a significant difference was defined as a p value < 0.05.

3. Results and Discussion

Such as the production of bacteriocin is dependent on the type of nutrient sources present in the medium (Todorov, & Dicks, 2004) and to confirm the unsuitability of this *P. pentosaceus* strain to use sucrose and inulin as the only carbon source, as demonstrated by Azevedo, Converti, Domínguez and Oliveira (2017), these both carbon sources were used to supplement the MRS medium. As a result, glucose is the most important carbon source that *P. pentosaceus* ATCC 43200 metabolizes.

The antimicrobial activity of bacteriocin extract produced by *P. pentosaceus* grown in different culture media was assessed by agar well diffusion assay, performed on Mueller Hinton and MacConkey (*E. coli)* agar-media, after 24 h cultivation against *Enterococcus* spp. (strains Ent101, Ent104 and Ent711), *Listeria innocua* (strains Li2052 and Li2865) and *E. coli* ATCC 25922, taken as examples of Gram-positive and Gram-negative microorganisms. *L. innocua* strains were sensitive to the cultivation supernatant of GI-MRS and GS-MRS collected after 24 h (21-22 mm diameter), about 26% larger than supernatants of other media. *E. coli* was sensitive to the cultivation supernatant of G-MRS, S-MRS and I-MRS media (17.3 mm), on average 6.5% greater than Ent711 to the same supernatant (Table 1). Figure 1 demonstrated the aspect of the inhibition halos

of bacteriocin extract produced by *P. pentosaceus* in G-MRS after 24 h of cultivation when the assays were performed on Mueller Hinton and MacConkey agar-media.

The reproducibility of these results performed on Muller Hinton and MacConkey agar-media (Table 1; Figure 1) was also assessed by comparing the activity of these supernatants performed on BHI and TSB agar-media (Table 2; Figure 2). It was evident the difference between the aspect and the diameters of the inhibition halos according to the agar-media used in the test. *Enterococcus* and *Listeria* strains were susceptible to the antimicrobial activity of supernatants (24 h) but the aspect and the diameters of the halos were not related to Muller Hinton and BHI agar-media. As expected, there was no antimicrobial activity against *E. coli* (Table 2) when the test was performed on TSB agar-medium, since Gram-negative bacteria are naturally resistant to the action of bacteriocins produced by Gram-positive bacteria due to their outer membrane (Cao-Hoang, Marechal, Le-Thanh, 2008; Gyawali, & Ibrahim, 2014). However, a questionable result could have been considered if this analysis had been carried out only on MacConkey agar-medium, since apparently inhibition halos against *E. coli* were seen only in MacConkey and were not seen in TSB agar-medium.

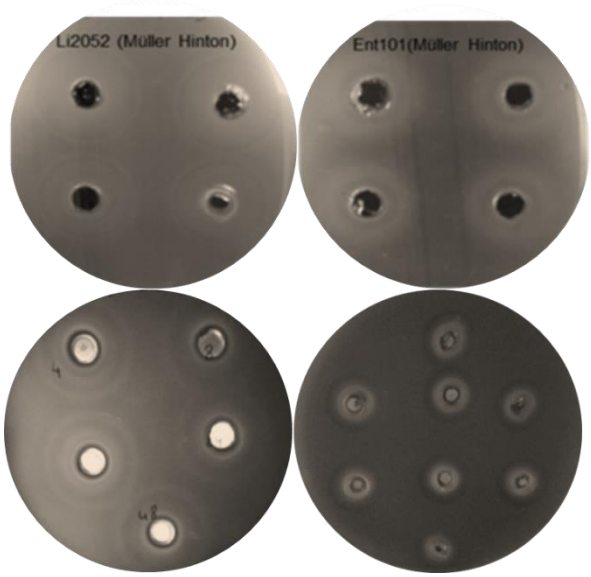

Figure 1. Antimicrobial activity of bacteriocin extract produced by *Pediococcus pentosaceus* ATCC 43200 after 24 h of cultivation in G-MRS medium against *Listeria innocua* 2052 (Li2052), *Enterococcus* 101 (Ent101) and *Escherichia coli* ATCC 25922 (*E. coli*). Tests were performed by agar-well-diffusion assay carried out on Muller Hinton (*Enterococcus* and *Listeria* strains) and MacConkey (*E. coli*) agar-media. (A, C, E = indicator strain at OD 0.3); (B, D, F = indicator strain diluted 100 times from OD 0.3).

Table 1. Antimicrobial activity (mm) of bacteriocin extract produced by *P. pentosaceus* ATCC 43200 cultivated in different media and collected at 24 h. The assays were performed on Mueller Hinton (*Enterococcus* and *Listeria*) and MacConkey (*E. coli*) agar-media

		Antimicrobial activity					
Time (h)	**Indicator strains**	G-MRS	GS-MRS	GI-MRS	S-MRS	I-MRS	GSI-MRS
24	*Enterococcus sp*. 101	12.10 ± 0.14^{a}	12.05 ± 0.07^{a}	12.00 ± 0.02^{a}	11.60 ± 0.14^{a}	11.00 ± 0.02^{a}	13.45 ± 0.07^{d}
	Enterococcus sp. 104	12.65 ± 0.07^{b}	13.35 ± 0.07^{b}	13.20 ± 0.14^{c}	13.90 ± 0.14^{b}	13.00 ± 0.03^{b}	12.55 ± 0.07^{c}
	Enterococcus sp. 711	15.40 ± 0.14^{c}	12.05 ± 0.07^{a}	12.00 ± 0.02^{a}	16.40 ± 0.14^{d}	16.60 ± 0.14^{e}	12.00 ± 0.02^{a}
	Listeria innocua 2052	16.50 ± 0.01^{d}	22.00 ± 0.04^{d}	20.05 ± 0.07^{d}	16.00 ± 0.11^{c}	15.00 ± 0.04^{c}	16.00 ± 0.04^{f}
	Listeria innocua 2865	18.00 ± 0.02^{e}	20.00 ± 0.03^{c}	21.35 ± 0.07^{e}	17.00 ± 0.04^{e}	16.00 ± 0.05^{d}	13.90 ± 0.14^{e}
	Escherichia coli ATCC 25922	16.60 ± 0.14^{d}	12.10 ± 0.14^{a}	13.00 ± 0.05^{c}	17.00 ± 0.04^{e}	18.45 ± 0.07^{f}	12.50 ± 0.01^{b}

Legend: G-MRS = glucose-based MRS medium, GS-MRS = glucose + sucrose-based MRS medium, GI-MRS = glucose + inulin-based MRS medium, S-MRS = sucrose-based MRS medium, I-MRS = inulin-based MRS medium, GSI-MRS = glucose + sucrose + inulin-based MRS medium. Mean values (n = 3) ± standard deviations. Different letters in the same column mean that values significantly differ among them ($p < 0.05$).

Table 2. Antimicrobial activity (mm) of bacteriocin extract produced by *P. pentosaceus* ATCC 43200 cultivated in different media and collected at 24 h. The assays were performed on BHI (*Enterococcus* and *Listeria*) and TSB (*E. coli*) agar-media

		Antimicrobial activity					
Time (h)	**Indicator strains**	G-MRS	GS-MRS	GI-MRS	S-MRS	I-MRS	GSI-MRS
24	*Enterococcus sp*. 101	17.95 ± 0.07^{b}	17.05 ± 0.07^{d}	17.00 ± 0.02^{e}	4.60 ± 0.14^{a}	4.05 ± 0.07^{a}	17.70 ± 0.14^{c}
	Enterococcus sp. 104	16.15 ± 0.21^{a}	16.10 ± 0.14^{c}	16.00 ± 0.01^{c}	7.15 ± 0.21^{b}	6.00 ± 0.01^{b}	16.15 ± 0.21^{a}
	Enterococcus sp. 711	16.00 ± 0.12^{a}	16.15 ± 0.21^{c}	16.25 ± 0.35^{d}	9.70 ± 0.01^{d}	9.65 ± 0.07^{f}	16.00 ± 0.02^{a}
	Listeria innocua 2052	16.40 ± 0.34^{a}	15.00 ± 0.01^{b}	13.15 ± 0.21^{a}	9.10 ± 0.14^{c}	8.05 ± 0.07^{c}	16.00 ± 0.02^{a}
	Listeria innocua 2865	16.45 ± 0.17^{a}	13.00 ± 0.02^{a}	14.40 ± 0.14^{b}	10.10 ± 0.14^{e}	9.00 ± 0.02^{e}	15.05 ± 0.07^{b}
	Escherichia coli ATCC 25922	-	-	-	-	-	-

Legend: G-MRS = glucose-based MRS medium, GS-MRS = glucose + sucrose-based MRS medium, GI-MRS = glucose + inulin-based MRS medium, S-MRS = sucrose-based MRS medium, I-MRS = inulin-based MRS medium, GSI-MRS = glucose + sucrose + inulin-based MRS medium. Mean values ($n = 3$) ± standard deviations. Different letters in the same column mean that values significantly differ among them ($p < 0.05$).

Among all strains evaluated, there was significant difference in the size of the inhibition halos according to the culture media used for the bacteriocin extract production (Table 1; Table 2) however, the most noteworthy observation that should be highlighted was the absence of inhibition halo against *E. coli* when the bacteriocin extracts were evaluated on TSB agar-medium.

A possible explanation for these results is the different agar-media composition. There are cations (Ca^{2+} and Mg^{2+}) and thymidine in the composition of Mueller Hinton agar-medium, components that are absent in BHI agar-medium, which may have somehow interacted with molecules occurring in the tested supernatants, affecting the obtained results (diameter of inhibition halo). MacConkey is a selective medium for Gram-negative bacteria and, unlike the TSB medium, there are bile salts, neutral red and crystal violet in its composition. In some way, the tested supernatants could interact with one of these different components present in MacConkey agar-medium, hence producing false positive results as shown in Figure 2.

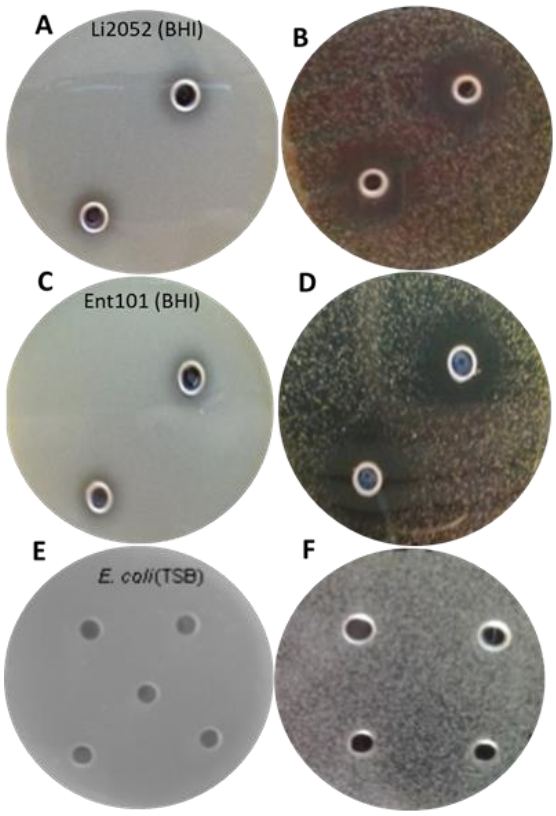

Figure 2. Antimicrobial activity of bacteriocin extract produced by *Pediococcus pentosaceus* ATCC 43200 after 24 h of cultivation in G-MRS medium against *Listeria innocua* 2052 (Li2052), *Enterococcus* 101 (Ent101) and *Escherichia coli* ATCC 25922 (*E. coli*). Tests were performed by agar-well-diffusion assay carried out on BHI (*Enterococcus* and *Listeria* strains) and TSB (*E. coli*) agar-media. (A, C, E = indicator strain at OD 0.3); (B, D, F = indicator strain diluted 100 times from OD 0.3).

Nowadays, it is difficult to find comparison in the literature about using bioassays performed only in solid (agar) medium as presented in this work, meanwhile it is possible to find more easily tests performed either in solid (agar diffusion assay) or in liquid (turbidimetry) media. A major difficult in bacteriocin research is obtaining accurate results using bioassays, which are based in inhibition activity produced in a sensitive microorganism (Rasch, & Knochel, 1998; Papagianni, Avramidis, Filioussis, Dasiou, & Ambrosiadis, 2006).

Using the agar spot assay, a method also performed on agar-medium and very similar to agar well diffusion assay, Arena et al. (2016) identified strong inhibition effect of seventeen *Lactobacillus plantarum* strains according to the classification made by Gaudana, Dhanani and Bagchi (2010), showing inhibition halos of more than 5 mm against the majority of food pathogens tested. The antimicrobial activity of the tested *Lb. plantarum* strains were mostly observed when they were grown on solid media than brought into contact with pathogenic bacteria. To performed well diffusion assay, the antimicrobial capability was confirmed when the cell free supernatant (CFS) was 10x concentrated, thus indicating that a minimal concentration of antimicrobial compounds was required to sustain similar inhibition results (Arena et al., 2016).

The CFS may also include other molecules besides those secreted by bacteria such as medium components and/or intracellular compounds, which may be accidentally released during CFS preparation (Arena et al., 2016). Despite this possibility, CFS are routinely used to preliminary screen of antimicrobial activity of bacteria by well diffusion method (Kassaa, Hober, Hamze, Chihib, & Drifer, 2014; Wang et al., 2014). This indicates that the inhibitory effects mainly depend on exudates, which are included in the CFS (Arena et al., 2016).

Other investigations observed that indicator cultures showed different sensitivity in agar and in liquid medium. The results of these investigations suggested a greater capability of CFSs to contrast pathogenic bacteria in liquid-medium than in agar-medium (Saadatzadeh, Fazeli, Jamalifar, Dinarvand, 2013). Compared with *Lactobacillus curvatus*, *Listeria monocytogenes* and *Listeria seeligeri* were more sensitive to bacteriocin action on agar-medium than in liquid-medium and bacteriocins showed a greater activity against *Lb. curvatus* in liquid-medium than in agar-medium (Coventry et al. 1997). In other investigation with nine test-microorganisms used, only two of them, *Lb. curvatus* ATCC 51436 and *P. acidilactici* ATCC 25740, were sensitive to very low bacteriocin (nisin) concentrations and produced a linear type of response either in agar-

medium (agar diffusion assay) or liquid-medium (turbidometric assay) (Papagianni, Avramidis, Filioussis, Dasiou, Ambrosiadis, 2006).

4. Conclusion

The results presented in this work indicated different measurements of bacteriocin activity to the same test-microorganisms when the assays were performed in different solid medium (agar diffusion assay). These data suggest that any comparison data in the literature must always be done among identical methods and highlights the need to carefully choose the agar-medium to be use in bacteriocin research.

CHAPTER III[1]

Effect of pH and carbohydrate sources on growth and antimicrobial activity of a bacteriocin-producing strain of *Pediococcus pentosaceus*

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ABSTRACT

The influence of pH as well as sucrose and inulin addition was investigated on the growth of *Pediococcus pentosaceus* ATCC 43200, a promising bacteriocin-producing strain. It was grown at pH 5.0 or 6.0 either in glucose-based MRS medium as such (control) or after addition of 0.5, 1.0 or 1.5% (w/w) sucrose and inulin (GSI-MRS) in the same percentages. In the control medium at pH 5.0, cell mass concentration after 48 h of fermentation (X_{max} = 2.26 g/L), maximum specific growth rate (μ_{max} = 0.180 h^{-1}) and generation time (T_g = 3.84 h) were statistically coincident with those obtained in supplemented media. At pH 6.0 some variations occurred in these parameters between the control medium (X_{max} = 2.68 g/L; μ_{max} = 0.32 h^{-1}; T_g = 2.17 h) and the above supplemented media (X_{max} = 1.90, 2.52 and 1.86 g/L; μ_{max} = 0.26, 0.33 and 0.32 h^{-1}; T_g = 2.62, 2.06 and 2.11 h, respectively). Bacteriocin released in the control medium at pH 5.0 displayed an antimicrobial activity against *Enterococcus* 101 5.3% larger than that at pH 6.0 and even 20% larger than those induced by cell free supernatants from all supplemented media, regardless of the pH and the concentration of supplements.

Keywords: *Pediococcus pentosaceus*, bacteriocin, probiotic, prebiotic

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1. Introduction

Pediococcus pentosaceus is a Gram-positive bacterium arranged in tetrads (Zhang et al. 2005) belonging to the group of lactic acid bacteria (LABs). It is the best-known major member of probiotic bacteria (Gibson and Fuller 2000; Rolfe 2000). Research and development with LABs has a great interest for applications in food preservation, agriculture, clinical use and environmental science (Cleveland et al. 2001) due to their ability to produce food additives and flavoring compounds (Gibson and Fuller 2000; Kothari et al. 2011). For these reasons, *P. pentosaceus* has been widely used in fermentation of vegetables, meats, silage and in cheese production (Simpson and Taguchi 1995).

P. pentosaceus ATCC 43200, previously denominated as *P. pentosaceus* FBB61, was isolated from fermented cucumber (Costilow et al. 1956) and its inhibitory activity (Etchells et al. 1964) was classified as bacteriocin-like (Rueckert 1979) with bactericidal effect (Piva and Headon 1994). Bacteriocin is defined as ribosomally-synthesized antimicrobial peptides with broad spectrum of action (Heng et al. 2007), especially against bacteria genetically closely related to the producer strain (De Vuyst and Vandamme 1994).

Several bacteriocins produced by *Pediococcus* spp. have been described, among which are *P. pentosaceus* FBB61 pediocin A (Etchells et al. 1964; Fleming et al. 1975), *Pediococcus acidilactici* PAC1.0 pediocin PA-1(Gonzales and Kunka 1987), *P. acidilactici* H pediocin AcH (Bhunia et al. 1987; Bhunia et al. 1988), *P. acidilactici* JD-23 pediocin JD (Richter et al. 1989), *P. acidilactici* SJ-1 pediocin SJ-1 (Schved et al. 1993), *P. pentosaceus* pediocin N5p (Strasser de Saad et al. 1995), *P. acidilactici* UL5 pediocin 5 (Huang et al. 1996), *Pediococcus damnosus* NCFB 1832 enterocin L50 (Cintas et al. 1998) and pediocin PD-1 (Nel et al. 2001).

Bacteriocin production was associated with the presence of plasmids (Gonzales and Kunka 1987) in strains of *P. pentosaceus* (Daeschel and Klaenhammer 1985) and may be improved by the nutrients available in the culture medium (Todorov and Dicks 2004) such as carbon sources (glucose, sucrose) and prebiotics (inulin). *P. pentosaceus* has the ability to ferment sucrose (Soro-Yao et al. 2014; Shukla and Goyal 2014) and inulin (Sabo et al 2015), the prebiotic used in this work, which is recognized as Generally Regarded as Safe (GRAS) food additive (Wagner et al. 2008; Holub et al. 2010) and exerts a stimulatory effect on the growth of probiotics (Roberfroid 2007) due to the

increased amount of fructose available from the glycolytic pathway (Oliveira et al. 2012). Since the antimicrobial activity is often related to the growth of the producing bacterium, inulin, as an ingredient of synbiotic media (containing both probiotic and prebiotic) (Gibson and Roberfroid 1995), might stimulate not only the probiotic growth, but also bacteriocin production (Sabo et al. 2015).

This work deals with the influence of initial pH and the amounts of sucrose and inulin as supplements on the growth of *P. pentosaceus* ATCC 43200 in glucose-based Man, Rogosa and Sharpe broth, sugar consumption, lactate production and bacteriocin activity.

2. Material and Methods

2.1. Bacteriocin-producing strain and growth conditions

Pediococcus pentosaceus ATCC 43200 used as bacteriocin producer was cultivated in Man, Rogosa, and Sharpe (MRS) medium (Difco Laboratories, Detroit, USA), pH 6.5 ± 0.2, for 16 h at 37ºC under 100 rpm agitation in orbital shaker, model TE-424 (Tecnal, Piracicaba, SP, Brazil).

2.2. Indicator strains and growth conditions

Enterococcus 101 (EN101) and *Escherichia coli* ATCC 25922 were used as indicator strains. To this purpose, they were cryopreserved at -70ºC in the presence of 20% (v/v) glycerol, and 1.0 mL of their suspensions was reactivated in 5.0 mL of Brain Heart Infusion (BHI) medium and in Tryptic Soy Broth (TSB) (Difco), respectively, for 16 h at 37ºC without agitation. To perform all assays, optical density (OD_{600nm}) of the indicator strains cultures was adjusted with sterile deionized water to an OD of 0.6 which corresponds to 2.6×10^{10} CFU/mL for EN101 and 2×10^{10} CFU/mL for *E. coli*.

2.3. Culture media

The MRS medium (Difco), that contains 20 g/L glucose, was used as the control medium after adjusting the pH to 5.0 or 6.0 with 1.0 N NaOH. The supplemented medium

(GSI-MRS) was prepared adding to the MRS medium 0.5, 1.0 or 1.5% (w/w) sucrose and inulin in the same proportion. Non-containing glucose media were prepared as the MRS medium, but the same amount of glucose was replaced by sucrose (S-MRS) or inulin (I-MRS) All media sterilization was performed in autoclave, model SD-75 (Phoenix Luferco, Araraquara, SP, Brazil), at 121ºC for 15 min.

2.4. Inoculum preparation and fermentation

P. pentosaceus pre-inoculum was prepared with 1.0 mL of cryopreserved culture inoculated into 50 mL of MRS medium, pH 6.5 ± 0.2, at 37ºC and under agitation (100 rpm) for 16 h. After the achievement of 10^7-10^8 CFU/mL, 10.0 mL of such a pre-inoculum were transferred to 250 mL Erlenmeyer flasks containing 100 mL of MRS medium at pH 5.0 or 6.0 (control) or of supplemented MRS media (0.5, 1.0 or 1.5%) at the same pH. Flasks were incubated in a rotatory shaker (100 rpm) at 30ºC for up to 48 h of fermentation. Samples (3.5 mL) to follow *P. pentosaceus* growth kinetics were collected every 2 h during the first 12 h and then after 24 and 48 h of fermentation. To assess the bacteriocin activity, samples were collected only after 4 h of fermentation. Cultures were performed in triplicate.

2.5. Determination of bacteriocin activity

Culture samples collected after 4 h of fermentation were centrifuged at 4470 *g* at 4ºC for 15 min. The pH of cell free supernatants (CFSs) was adjusted to 6.0-6.5 by addition of 1.0 N NaOH, heated at 70ºC for 25 min to inactivate proteases and sterilized with 0.45 µm-pore diameter filters (Millipore, USA). The CFS inhibitory activity, determined as diameter of the inhibition halo, were tested on EN101 and *E. coli* by the spot-on-lawn method. Briefly, one hundred µL of each indicator strain (OD = 0.6) were added into 10 mL of melted agar-medium (1.0%; w/w) and poured into Petri dishes, which were incubated at 37ºC for 18 h.

2.6. Analytical procedures

During the fermentation process, *P. pentosaceus* cell mass concentration was determined by optical density measurements using a calibration curve (R^2 = 0.997) of OD_{600nm} versus dry weight (DW), expressed in g_{DW}/L, while the progressive acidification of the culture media was followed through a pHmeter, model 400M1 (Quimis, Brazil). Glucose, sucrose and lactate concentrations were determined by a High Performance Liquid Chromatograph (HPLC) LC-20A Prominence (Shimadzu, Kyoto, Japan). Analyses were performed in triplicate, at room temperature using 75:25% acetonitrile:ultrapure water as mobile phase (0.9 mL/min) and high purity glucose and sucrose (Sigma-Aldrich, USA) as standard solutions.

2.7. Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's test from Statistica Software 13.3 (TIBICO Software Inc., Palo Alto, CA, USA) was used, considering a significant difference for values of $p < 0.05$.

3. Result and Discussion

3.1. Cell growth, yield of biomass and lactate production

The growth of *P. pentosaceus* ATCC 43200 was followed at two pH values (5.0 and 6.0), in order to determine the lag phase duration. These pH values were selected based on previous results of the authors that showed better growth of *P. pentosaceus* ATCC 43200 (results not shown). Under both conditions, the microorganism was able to grow either in glucose-based MRS medium without any supplement, selected as control, or in all media supplemented with sucrose and inulin (Figure 1). However, when the initial pH of the control medium was 6.0 it started to grow after 6 h, while at the lower pH (5.0) the lag phase was about 4 h longer.

Such an influence of initial medium pH on the duration of *P. pentosaceus* lag phase agrees with other results reported in the literature. For instance, when this microorganism was cultivated in medium at pH 4.3, the lag phase was as long as 25 h (Blickstad, & Molin, 1981). Abbasiliasi et al. (2012) also identified impaired survival of

Pediococcus acidilactici Kp10 under acidic conditions, in that percent survival at pH 3.0 was >97% after 1 to 3 h incubation; however, after 4 h there was no further growth.

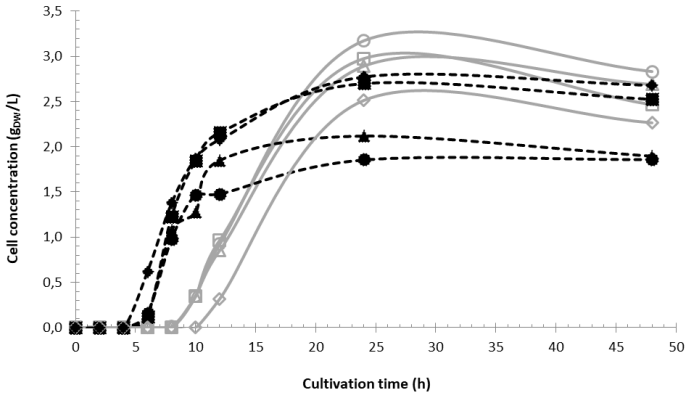

Figure 1. Growth curves at pH 5.0 (solid line and empty grey symbol) and 6.0 (dotted lines and full black symbol) of *Pediococcus pentosaceus* ATCC 43200 in MRS medium selected as control (◇,◆), in MRS media supplemented with (△,▲) 0.5%, (□,■) 1.0%, and (○,●) 1.5% sucrose and inulin. Average of runs carried out in triplicate (n = 3).

As far as the supplementation of sucrose and inulin in the glucose-based MRS medium (GSI-MRS) is concerned, both supplements exerted a positive influence on the lag phase. When the initial medium pH was 5.0, *P. pentosaceus* did in fact reach the exponential growth after 12 h in the control medium and 2 h before in the supplemented ones, regardless of the concentration of these supplements. This result demonstrates not only the ability of these ingredients to speed up *P. pentosaceus* ATCC 43200 adaptation to pH 5.0, but also the potential synbiotic effect of this probiotic in combination with a prebiotic (inulin) and with more than one carbon source (glucose and sucrose). On the other hand, at pH 6.0 there was no difference in the lag phase duration between fermentations carried out in control medium or in supplemented media, thereby pointing out that this may be the optimum pH for the growth of this microorganism.

After 48 h of fermentation, cell mass concentration (X_{max} = 2.26 g/L dry weight) in the control medium at pH 5.0 was statistically coincident ($p > 0.05$) with those obtained supplementing sucrose and inulin up to the three selected levels (0.5, 1.0 and 1.5%) (X_{max} = 2.69, 2.46 and 2.83 g/L dry weight, respectively). As a result, maximum specific growth rate (μ_{max} = 0.18 h^{-1}) and generation time (T_g = 3.84 h) in the control were equally statistically coincident ($p > 0.05$) to those in supplemented media (μ_{max} = 0.18, 0.18 and 0.19 h^{-1}; T_g = 3.86, 3.76 and 3.50 h, respectively), confirming that glucose is the carbon source preferred by this microorganism and that, in its presence, sucrose and inulin

addition, even in different concentrations, did not exert any effect. However, when the initial pH was 6.0, cell mass concentration after the same time was different between the control (X_{max} = 2.68 g/L dry weight) and the same supplemented media (X_{max} = 1.90, 2.52 and 1.86 g/L dry weight, respectively) ($p < 0.05$), even though no regular dependence of this parameter could be observed. On the other hand, important insights came from maximum specific growth rate (μ_{max} = 0.32, 0.26, 0.33 and 0.32 h^{-1} respectively) and generation time (T_g = 2.17, 2.62, 2.06 and 2.11 h, respectively), which were, on average, 68% higher and 40% shorter, respectively, compared with those observed at pH 5.0, hence confirming 6.0 as the optimum pH value. Perez, Perez and Elegado (2015) attributed to pH a similar importance on the growth of *P. acidilactici* NRRL B-5627 in whey, supplemented or not with glucose and yeast extract.

It is noteworthy that glucose replacement by only sucrose (S-MRS) or only inulin (I-MRS) almost completely suppressed growth as well as lactate and bacteriocin productions by *P. pentosaceus*, regardless of the initial medium pH (results not shown), indicating that these ingredients were not effectively uptaken as the only carbon source and providing a further confirmation that glucose is the preferable carbon source for this strain. Therefore, these media will not be taken into further account in this section.

The yield of lactate on consumed glucose ($Y_{P/S}$) either in the medium at pH 5.0 or in that at pH 6.0 is illustrated in Figure 2, panels A and B, respectively. Irrespectively of the initial pH, *P. pentosaceus* was able to start lactate production after 2 h of fermentation. However, at pH 5.0 the addition of only 0.5% sucrose and inulin was able to favor lactate production at the beginning of fermentation (2 h), while higher amounts (1.0 and 1.5%) did so much later (10 h). On the other hand, when the initial pH was 6.0, lactate production decreased significantly after 6 h in the control medium, whereas increased in all supplemented media (GSI-MRS). As expected by the competition between cell growth and product formation, such a decrease (Figure 2, panel B) was accompanied by a simultaneous remarkable increase in cell growth (Figure 3, panel B). These results suggest that the *P. pentosaceus* strain used in the present work may have been capable of directly metabolizing the extra carbon source (sucrose) and some of the prebiotic (inulin) when present in an unfavorable environment like that existing in the medium at pH 5.0.

A behavior opposite to that of $Y_{P/S}$ was observed for the biomass yield on consumed glucose ($Y_{X/S}$), which strongly depended on the initial medium pH (5.0 or 6.0). At pH 5.0 the addition of supplements to MRS broth remarkably increased this parameter after 10 h (Figure 3, panel A), whereas no growth improvement was observed at pH 6.0 (Figure 3, panel B).

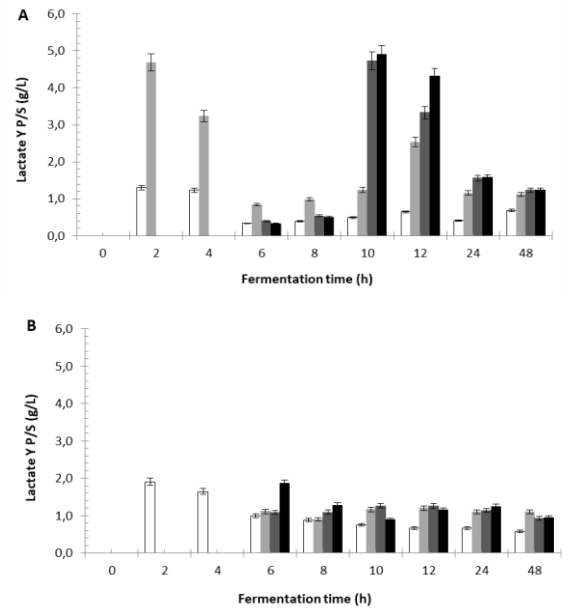

Figure 2. Yields of lactate on consumed glucose in MRS medium selected as control (☐), in MRS media supplemented with (▒) 0.5%, (▓) 1.0%, and (■) 1.5% sucrose and inulin. pH: (A) 5.0; (B) 6.0. Average of runs carried out in triplicate (n = 3).

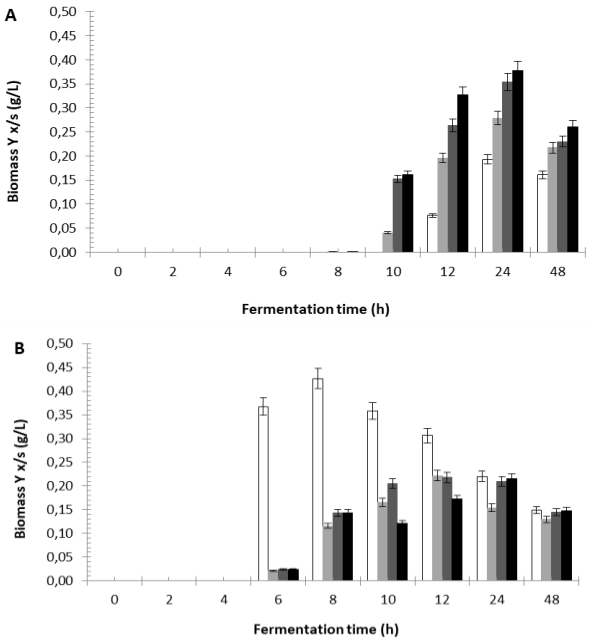

Figure 3. Yields of biomass on consumed glucose in MRS medium selected as control (☐), in MRS media supplemented with (▒) 0.5%, (▓) 1.0%, and (■) 1.5% sucrose and inulin. pH: (A) 5.0; (B) 6.0. Average of runs carried out in triplicate (n = 3).

3.2. Acidification and lactate production

The acidification profiles in the control medium and in all supplemented (0.5, 1.0 and 1.5%) media (GSI-MRS) were statistically coincident ($p > 0.05$), showing gradual pH decreases from 5.0 at the beginning to 3.5 (control) or 3.7 (GSI-MRS) and from 6.0 at the beginning to 3.8 (control) or 3.9 (GSI-MRS) at the end of fermentations (Figure 4). Such an acidification profile of *P. pentosaceus* is an interesting feature in view of ingesting this strain as a probiotic, because it may contribute to decrease the colonic pH and then to prevent the proliferation of non-acid-tolerant bacteria, food-borne pathogens and spoilage organisms (Adams, & Hall, 1988; Holyoak et al., 1996; Cintas, Casaus, Herranz, Nes, & Hernández, 2001), forcing them to utilize the remaining energy to oust excess proton (Holyoak et al., 1996).

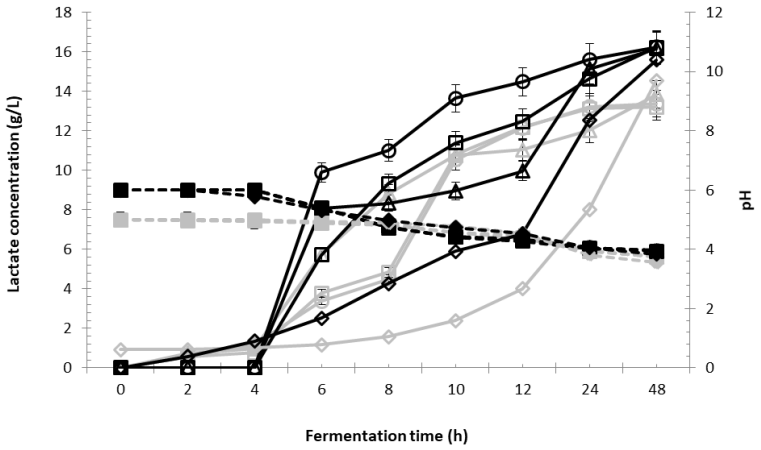

Figure 4. Lactate production at pH 5.0 (solid line and empty grey symbol) and 6.0 (empty black symbols) by *Pediococcus pentosaceus* ATCC 43200 in MRS medium selected as control (◇,◇), in MRS media supplemented with (△,△) 0.5%, (□,□) 1.0%, and (○,○) 1.5% sucrose and inulin. Corresponding acidification profiles after 48 h at pH 5.0 (dotted line and full grey symbol) and 6.0 (full black symbols), in media with only one carbon source: only glucose (◆,◆) G-MRS, only sucrose (▲,▲) S-MRS, and only inulin (■,■) I-MRS.

As shown in the same figure, lactate production was remarkable at both pH values (5.0 and 6.0) either in controls or in all GSI-MRS media. However, in these fortified media lactate production after 48 h of fermentation was, at pH 5.0 and 6.0, 1.3-fold higher (13.0 g/L) and 1.5-fold higher (16.0 g/L) than in their respective control media (9.7 and 10.4 g/L), which highlights the beneficial effect of sucrose and inulin also from lactate production viewpoint, regardless of the concentration of these ingredients.

Even though lactate production was certainly the main event responsible for acidification, it has been reported that prebiotics such as inulin are capable of reducing

pH through an indirect mechanism due to the production of other products such as bacteriocins (Dunkley et al., 2009; Rehman, Vahjen, Kohl-Parisini, Ijaz, & Zentek, 2009) and/or other organic acids (Rémésy, Levrat, Gamet, & Demigné, 1993).

3.3. Glucose and sucrose consumption

After 48 h, the consumption of glucose, whose starting concentration was 20.0 g/L in all the fermentations, was always higher in control media, where it was the only sugar, compared with all GSI-MRS media. In control media, it was in fact as high as 70.3 and 89.9% at pH 5.0 and 6.0 ($p < 0.05$), respectively, while in 0.5, 1.0 and 1.5% GSI-MRS media it was, on average, only 56.6 ± 4.6% ($p > 0.05$) at pH 5.0 and no less than 82.1 ± 7.9% ($p > 0.05$) at pH 6.0 (results not shown). Such a 45.0% increase in glucose consumption by *P. pentosaceus* in supplemented media at pH 6.0 compared with pH 5.0 confirms the importance of using an optimal pH also for this response.

As regards sucrose consumption in fortified media, there was no significant difference among them, being about 1.0% as an average (results not shown).

3.4. Bacteriocin activity of culture supernatants

The antimicrobial activity of cell free supernatants (CFSs) was assessed against EN101 and *E. coli* only after 4 h of fermentation, because a preliminary kinetic study on *P. pentosaceus* metabolism (results not shown) revealed that this microorganism was able to release a bacteriocin at the highest concentration just in the late lag phase. Table 1 shows that the diameter of EN101 inhibition halo (12.0 mm) induced by the CFS from the control medium at pH 5.0 was 5.3% larger than that at pH 6.0 (11.4 mm) and even 20% larger than those induced by CFSs from all supplemented media, regardless of the pH and the concentration of supplements ($p > 0.05$). The CFS showed no antimicrobial activity against *E. coli* (results not shown).

Consistently with these results, Singh et al. (2014) observed that the activity of *P. pentosaceus* IE-3 bacteriocin was maximal at pH 5.0 and suffered significant loss at pH 8.0, while Mathys et al. (2007) observed loss of *P. pentosaceus* UVAI pediocin activity at pH ≥ 10.

Table 1. Antimicrobial activity of cell-free supernatants against *Enterococcus sp.* 101 (EN101), collected after 4 h from different media fermented by *Pediococcus pentosaceus* ATCC 43200. The results are expressed as diameter of inhibition halo (mm)

pH	Control	GSI-MRS (0.5%)	GSI-MRS (1.0%)	GSI-MRS (1.5%)
5.0	12.0 ± 0.1^{c}	9.9 ± 0.1^{a}	10.2 ± 0.2^{a}	9.9 ± 0.1^{a}
6.0	11.4 ± 0.0^{b}	10.1 ± 0.1^{a}	9.9 ± 0.1^{a}	9.8 ± 0.2^{a}

Legend: GSI-MRS = MRS media supplemented with sucrose and inulin in different proportions. Mean values (n = 3) ± standard deviations. Different letters in the same column mean statistically significant difference among the values of the same parameter, according to the test of Tukey ($p < 0.05$).

These results as a whole demonstrate that bacteriocin production was favored either under suboptimal environmental conditions (pH lower than the optimum) or in the absence of any prebiotic able to stimulate growth.

The absence of any antimicrobial activity against *E. coli* is consistent with the higher resistance of the outer membrane of Gram-negative bacteria against the action of bacteriocin in comparison to the Gram-positive ones, which are naturally more susceptible. However, contradictory results can be found in the literature concerning bacteriocin activity against Gram-negative bacteria (Conventry et al., 1997; Ramírez, & Otálvaro, 2008).

Pranckuté et al. (2014) demonstrated that the ability to metabolize different carbon sources (e.g. sucrose, inulin) by *Lactobacillus* sp. and *Lactococcus* sp., on the production of bacteriocin, was strain specific and, in agreement with the results of this work, glucose was always the preferred carbon source; however, it was consumed more quickly by *Lactococcus lactis* rather than by *Lactobacillus* sp.; on the other hand, *Lactococcus* sp. assimilated poorly inulin, while *Lactobacillus* sp. were completely ineffective in this respect.

These results suggest that the bacteriocin produced by *P. pentosaceus* ATCC 43200 could be a promising alternative as preservative in food industry.

4. Conclusion

The metabolism of *Pediococcus pentosaceus* ATCC 43200, a promising bacteriocin-producing strain, was investigated at two different initial values of medium pH as well as in the presence or the absence of sucrose and inulin as supplements of the glucose-based MRS medium. The addition of sucrose and inulin to MRS improved growth and production of lactate by *P. pentosaceus*, whereas it affected bacteriocin production/activity. Even though this *Pediococcus* strain was able to grow either in the culture media with or without supplements, these showed a positive influence on the growth only when the initial pH of the medium was 5.0. Under these suboptimal conditions, *P. pentosaceus* growth was significantly delayed compared with the medium at pH 6.0. The initial pH of the medium influenced not only the duration of the lag phase, but also the cell growth and the productions of lactate and bacteriocin. Lactate production was remarkable at both pH values (5.0 and 6.0) either in controls or in all supplemented media. This production (13-16 g/L) was 1.3 to 1.5-fold higher than in their respective

control media, which highlights the beneficial effect of sucrose and inulin also on lactate production, regardless of the concentration of these ingredients. Bacteriocin activity against *Enterococcus* 101 exerted by the cell free supernatant from the control medium at pH 5.0 (12.0 mm) was larger than that at pH 6.0 and those of supernatants from all supplemented media. These results as a whole demonstrate that bacteriocin production was favored either under suboptimal environmental conditions or in the absence of any prebiotic able to stimulate growth.

CHAPTER IV[1]

Biotechnological production of bacteriocin-like substance by *Pediococcus pentosaceus* in MRS commercial medium supplemented with nitrogen source

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ABSTRACT

Bacteriocins are peptides produced by various species of bacteria, especially lactic acid bacteria (LABs), which exhibit a large spectrum of action against spoilage bacteria and foodborne pathogens. However, when they have not been completely characterized, the qualified term bacteriocin-like inhibitory substances (BLIS) is recommended. The best *Pediococcus pentosaceus* ATCC 43200 growth (3.41 g/L) and BLIS activity (19.50 mm) occurred after 24 h of cultivation at 30ºC in Man, Rogosa and Sharp (MRS) medium supplemented with 1.5% peptone under anaerobiosis and agitation of 200 rpm. Under such optimized conditions, the cell mass concentration was about 66% higher, generation time about 38% shorter and BLIS activity 30% higher than in MRS medium without any supplement taken as a control (2.05 g/L, 2.05 h, and 15.00 mm, respectively), and the exponential phase started 4 h before. The agar diffusion method showed BLIS inhibition halos against LABs strains with diameter in the range of 12.50 to 19.50 mm. However, BLIS antimicrobial activity against *Listeria* strains was better detected by the liquid medium assay, which showed 100 and 60% inhibition of *L. innocua* and *L. seeligeri* growth, respectively. These results demonstrate the potential of *P. pentosaceus* ATCC 43200 BLIS as an antimicrobial.

Keywords: *Pediococcus pentosaceus*, probiotic, BLIS, nitrogen source, antimicrobial activity

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1. Introduction

In the last years, due to the constant request of consumers for products free of chemical additives (Cortés-Zavaleta, López-Malo, Hernández-Mendonza, & García, 2014), the use of probiotic bacteria or their metabolites to increase food shelf life has become a growing and promising area of research. Therefore, research on biotechnological production of natural antimicrobials is expected to provide the food industry with new natural additives with potential application and broad spectrum of action against spoilage bacteria and foodborne pathogens.

Lactic acid bacteria (LABs) are Gram-positive bacteria producing high value-added bioproducts during their fermentation. Lactic acid is the major metabolite produced by LABs; however, other compounds (e.g. acetic acid, ethanol, diacetyl, acetone, proteases, bacteriocins and exopolysaccharides) can be synthesized, whose preservative characteristics can increase the shelf life of the final product.

LABs are recognized by the U.S. Food and Drug Administration (FDA) as Generally Regarding as Safe (GRAS) microorganisms, which can be used in the production of a wide variety of food additives and flavory compounds (Ly et al., 2008; Kothari, Tyagi, Patel, & Goyal, 2011). Some LABs belonging to the *Pediococcus* genus that colonize the gastrointestinal tract of mammals are considered probiotics, because they inhibit the growth of intestinal pathogens by stimulating the immune system, reducing total and LDL cholesterol levels and improving protein digestion, hence leading to an increase in the absorption of vitamins and minerals (Cheikhyoussef et al., 2010; Saad, Delattre, Urdaci, Schmitter, & Bressollier, 2013).

The *Pediococcus* genus is composed of Gram-positive homofermentative, motile, non-spore-forming and facultative anaerobic cocci (Zhang, Tong, & Dong, 2005), which are usually organized in tetrads. Moreover, they have complex nutritional requirements and limited respiratory capacity, and their multiplication is favored under microaerophilic and occasionally anaerobic conditions (Hough, Briggs, Stevens, & Young, 1982; Priest, 2006).

Bacteriocins are antimicrobial peptides produced by a wide variety of bacteria, including LABs, which have a broad spectrum of action against Gram-positive and Gram-negative bacteria, protozoa, fungi and viruses (Reddy, Yedery, & Gupta, 2004), thereby behaving as effective food biopreservatives (Galvez, Lopez, Abriouel, Valdivia, & Omar, 2008). Structurally, bacteriocins are composed of 20 to 60 amino acids, which are

responsible for their main properties, namely acid tolerance, thermostability and bactericidal specificity (Collins, Cotter, Hill, & Ross, 2010). Nisin, produced by many strains of *Lactococcus lactis* subs. *lactis*, is the most known bacteriocin, being considered the prototype of LABs bacteriocins (Perin, Moraes, Viçosa, Júnior, & Nero, 2012). When a bacteriocin has not yet been completely characterized in terms of its amino acid composition and nucleotide sequences of the corresponding gene, the term bacteriocin-like inhibitory substance (BLIS) is recommended (Tagg, Dajani, & Wannamaker, 1976; Jack, & Ray, 1995).

Many probiotic bacteria, including *Pediococcus* spp. (Saad et al., 2006), have been widely used in bioprocesses devoted to the production of foods such as vegetables, meats (Anastasiadou, Papagianni, Filiousis, Ambrosiadis, & Koidis, 2008) and dairy products (Drieder, Fimland, Héchard, McMullen, & Prévost, 2006). Specifically, bacteriocin-producing bacteria have been applied in the control of microorganisms during fermentation as well as the inhibition of spoilage bacteria growth during product storage (Díez et al., 2012).

To increase the antimicrobial activity of these peptides, the ability of probiotic LABs to ferment different carbon and nitrogen sources has been studied. In fact, to achieve the best performance and obtain the desired product, there is a need for selection of these sources so as to improve the process conditions and provide adequate supply to the cells for biosynthesis and energy generation (Chen, Kirk, & Piper, 1993). In addition, it should be taken in mind that feedstock is one of the most important factors influencing the cost of producing biomolecules by fermentation as well as the composition of the culture medium, which should be carefully defined (Lilly, 1979).

Natural preservatives such as bacteriocins and/or BLIS have been increasingly targeted by research because of their low toxicity and proven antimicrobial action able to inhibit the growth of spoilage and pathogenic microorganisms. In this context, this study opens the way to optimize the biotechnological production of BLIS by *Pediococcus pentosaceus* ATCC 43200 and to make possible the discovery of new antimicrobial molecules with potential as natural food preservatives.

2. Material and Methods

2.1. Optimization in rotary shaker

To optimize medium composition and culture conditions, *Pediococcus pentosaceus* ATCC 43200 cultivations were carried out for 24 h in rotary shaker (model TE-424, Tecnal, Piracicaba, SP, Brazil) on the commercial Man, Rogosa and Sharpe (MRS) (Difco Laboratories, Detroit, MI, USA) medium supplemented with peptone or urea as nitrogen source at different concentrations (0.5, 1.0 and 1.5%). To this purpose, we tested either microaerophilic conditions in flasks under 150 or 200 rpm agitation according to the fractional factorial design of Table 1 or anaerobic ones in jar. On the other hand, temperature (30^{o}C) and pH (6.0 ± 0.2) were previously optimized by our research-group (results not shown). Thereafter, according to the best results of 24 h cultivations, the kinetics of *P. pentosaceus* growth was investigated.

Table 1. Fractional factorial design used to optimize *Pediococcus pentosaceus* ATCC 43200 cultivations in shaken flasks and under anaerobic conditions

Variables	Code	-1	0	1
N source* (%)	x_1	0.5	1.0	1.5
Agitation (rpm)	x_2	150	200	Anaerobic

* N source: peptone or urea.

2.2. Batch cultivation in bioreactor

After the optimization in rotary shaker, the conditions that ensured the maximum BLIS activity were reproduced as much as possible in a larger scale in bioreactor (Biostat B® Sartorius, Göttingen, Germany) operated in batch mode.

2.3. BLIS producing strain

The BLIS producing strain *P. pentosaceus* ATCC 43200 was purchased from the American Type Culture Collection (ATCC). For the inoculum preparation, it was grown in MRS medium, pH 6.5 ± 0.2, at 37^{o}C for 16 h under agitation of 100 rpm in rotary shaker.

2.4. Indicator strains

To determine BLIS activity, the following indicator strains were used: *Listeria monocytogenes* NADC 2045, *Listeria monocytogenes* CECT 934, *Listeria innocua* NCTC 11288, *Listeria seeligeri* NCTC 11289, *Enterococcus faecium* 2052, *Enterococcus faecium* 2865, *Staphylococcus aureus* CECT 239, *Pseudomonas aeruginosa* CECT 116, *Salmonella enterica* CECT 724, *Escherichia coli* ATCC 25922, *Lactobacillus plantarum* CECT 221, *Carnobacterium piscicola* CECT 4020 and *Lactobacillus sakei* ATCC 15521.

To activate them, 1 mL of the cryopreserved culture at 70ºC in the presence of 20% glycerol (v/v) was added into 5 mL of Brain Heart Infusion (BHI) medium (Difco Laboratories, Detroit, MI, USA) for *Listeria*, Tryptic Soy Broth (TSB) for *S. aureus*, *P. aeruginosa*, *S. enterica* and *E. coli* and MRS for *L. plantarum*, *C. piscicola* and *L. sakei*. The cultures were incubated at 37ºC for 16 h without agitation and then diluted with sterilized deionized water up to an optical density at 600 nm (OD_{600mn}) of 0.3, corresponding to 3×10^8 CFU/mL for *L. monocytogenes*, 3×10^6 CFU/mL for *Enterococcus* sp., *L. innocua* and *L. seeligeri*, 1×10^8 CFU/mL for *S. aureus*, *P. aeruginosa* and *S. enterica*, 1×10^7 CFU/mL for *E. coli*, 6×10^6 CFU/mL for *L. plantarum* and *C. piscicola* and 8×10^6 CFU/mL for *L. sakei*.

2.5. Culture medium preparation

The MRS medium was prepared adding peptone or urea at the selected concentration, adjusting the pH to 6.0 ± 0.2 by the addition of 1.0 N HCl and autoclaving at 121ºC for 15 min. For the bioreactor runs, 1.5 L of MRS medium supplemented according to the best condition obtained on the rotary shaker was prepared and conditioned inside the bioreactor, and the whole system was autoclaved at 121ºC for 15 min.

2.6. Cultivation in shaken flasks

To prepare the pre-inoculum, 1 mL of the previously cryopreserved *P. pentosaceus* culture at 70ºC in the presence of 20% glycerol (v/v) was added into 125 mL Erlenmeyer flasks containing 50 mL of MRS medium, pH 6.5 ± 0.2, and placed in a rotary

shaker at 37ºC for 16 h under agitation of 100 rpm. The growth was monitored by OD_{600mn} of the culture medium until reaching OD of 0.8-0.9, corresponding to 10^7-10^8 CFU/mL. Ten mL of this pre-inoculum suspension were transferred to a 250 mL Erlenmeyer flask containing 100 mL of supplemented MRS medium, pH 6.0 ± 0.2, and placed in a rotary shaker at 30ºC under different stirring conditions (Table 1). To investigate *P. pentosaceus* growth kinetics, samples were collected in triplicate every 2 h during the first 12, 24 and 48 h.

To ensure anaerobic conditions, 50 mL polypropylene tubes were filled with 45 mL of the respective supplemented medium, completed with 5 mL of the above *P. pentosaceus* suspension (OD 0.8-0.9) and placed into anaerobic jars (BBL GasPack®System, BioQuest, Cockeysville, MD, USA) at 30ºC.

2.7. Cultivation in bioreactor

One hundred mL of the same pre-inoculum suspension used for cultures in shaken flasks (OD_{600nm} 0.8-0.9) were transferred to a 2 L bioreactor containing 1.5 L of MRS medium, pH 6.0 ± 0.2, supplemented with nitrogen source and stirred according to the best result obtained in shaken flasks. The culture temperature was maintained at 30ºC during the whole fermentation process, and samples were collected in triplicate every 2 h during the first 12, 24 and 48 h to study *P. pentosaceus* growth kinetics.

2.8. Kinetics of *P. pentosaceus* growth

The growth of *P. pentosaceus* was followed using a calibration curve ($R^2 = 0.997$) constructed plotting data of cell mass concentration versus OD_{600mn} of cell suspensions at different dilutions (5x, 6x, 7x, 8x, 9x, 30x). To determine cell mass concentration, the microorganism was cultivated at 30ºC for 16 h under agitation of 100 rpm, and 10 mL of each culture dilution were then filtered through membranes with 0.45 µm pore diameter (Millipore, Billerica, MA, USA). The membranes were dried at 100ºC for 2 h, kept in a desiccator for cooling without contact with outer humidity and weighed.

The pour plate technique was also used for colony counts during cultures (0-48 h). Plates were incubated at 37ºC for 24 h, and then colonies were counted.

2.9. BLIS antimicrobial activity

The BLIS antimicrobial activity was determined by the agar-diffusion method. To this purpose, each indicator strain suspension with OD_{600nm} of 0.3 was 100x (v/v) and 200x diluted with sterile deionized water for non-pathogenic and pathogenic strains, respectively. After dilutions, 150 µL of each strain suspension were added to 15 mL of soft agar-medium (0.75%, w/v) and poured into Petri dishes. After agar solidification, wells were made on the agar using tips and 50 µL of BLIS solution were added into each well. All plates were incubated for 16-18 h at 37ºC in duplicate. The antagonistic activity was determined by measuring the inhibition halos in millimeters.

2.10. BLIS-size determination by SDS-PAGE

The crude extract (i.e. BLIS without purification) was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The 12.5% gel (0.5 × 80 × 100 mm) was prepared with 10% acetic acid, and the electrophoretic run was performed with constant voltage of 30 mA for 2 h. After electrophoresis, the gel was silver stained (Merril, Goldman, Sedman, & Ebert, 1981) and washed with 10% acetic acid solution until obtaining clear revelation. To determine the size of the migrated fragments, a molecular weight marker of 2.0 – 100.0 kDa (Bio-Rad, São Paulo, Brazil) was used.

2.11. Amplification and sequencing of the 16S rDNA gene of BLIS-producing strain

The genomic DNA was extracted using the commercial kit Wizard®Genomic DNA Purification Kit (Promega Corporation, Fitchburg, WI, USA), and procedures were carried out according to the manufacturer's instructions. The 16S rDNA gene was amplified by Polymerase Chain Reaction (PCR) using the following primers: F5'GAGAGTTTGATCCTGGCTCAG3' and R5'CGGTGTGTACAAGGCCCGGGAA CG3'. DNA amplification was carried out in a volume of 25 µL, and the temperature profile of the reaction was as follows: after the denaturation step of 94ºC for 30 s, the annealing temperature was set at 60ºC for 30 s, and 35 additional cycles were carried out. Extension was performed at 72ºC for 10 min, and the final cycles were followed by an

additional step for 10 min at 72ºC. Following the amplification, 8 µL of solution were electrophoresed in 1.5% (w/v) agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) stained with SYBR®Safe (Thermo Fisher Scientific, Waltham, MA, USA) and photographed in UV light.

The sequencing of the PCR product was performed at USP Genome Center using the following primers: F5'GAGAGTTTGATCCTGGCTCAG3' and R5'CGGTGTGTA CAAGGCCCGGGAACG3'; F5'AACGCGAAGAACCTTAC3' and R5'CCGTCAATT CCTTTRAGTTT3'. The 16S rDNA sequence of *P. pentosaceus* ATCC 43200 and its phylogenetically closely related LAB were obtained from the PUBMED database.

2.12. BLIS minimal inhibitory concentration of 50%

To determine the minimal inhibitory concentration of BLIS required to inhibit the growth of the 50% organisms (MIC_{50}), crude BLIS (CB), which refers to BLIS produced after 10 and 24 h of bioreactor fermentation without purification, and powdered BLIS (PB) that refers to the lyophilized BLIS were analyzed. The indicator strain *L. sakei* was used, since the members of the *Lactobacillus* genus, especially *L. sakei* (Dykes, Britz, & Von Holly, 1994), are considered spoilage bacteria of vacuum-packed fresh meat products (Castellano, Holzapfel, & Viignolo, 2004). *L. sakei* was cultivated in MRS medium for 14 h at 37ºC under agitation of 130 rpm.

To obtain the control assay (OD_{600nm} 0.3), *L. sakei* culture was diluted with sterilized deionized water up to OD_{600nm} of 0.4, and 1.0 mL of the resulting suspension was diluted with 1.0 mL of sterile water. After dilution up to OD_{600nm} of 0.3, 1.0 mL of the resulting suspension was diluted with 1.0 mL of CB. *L. sakei* OD_{600nm} 0.3 suspension was then diluted several times with 1.0 mL of CB previously diluted (1 to 5000) with sterilized deionized water, as shown in Figure 1. After preparation of all dilutions, the tubes were incubated for 18 h at 37ºC under agitation of 200 rpm. For colony counting, the Pour plate method was applied as described above. The assay was performed in duplicate.

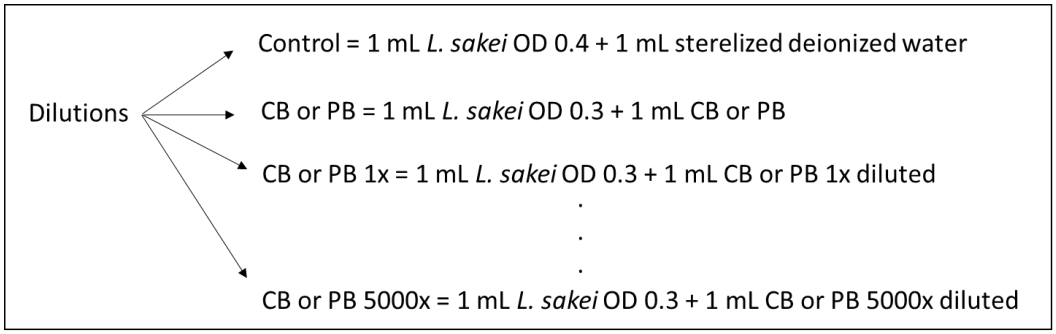

Figure 1. Schematic of BLIS minimal inhibitory concentration of 50% (MIC_{50}) assay. Legend: OD: optical density, *L. sakei*: *Lactobacillus sakei* ATCC 15521, CB: crude BLIS, PB: powdered BLIS.

2.13. Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's test were performed using the Statistica Software 13.3 (TIBICO Software Inc., Palo Alto, CA, USA). A significant difference was defined as a *P* value < 0.05.

3. Results and Discussion

3.1. Optimization of *P. pentosaceus* growth

Table 2 lists the results of *P. pentosaceus* cultivations carried out at 30°C for 24 h in supplemented MRS medium under the conditions listed in Table 1. Since the *Pediococcus* genus has limited breathing capacity and its multiplication is favored under microaerophilic conditions (Hough, Briggs, Stevens, & Young, 1982; Priest, 2006), Table 2 lists the results of *P. pentosaceus* cultivations carried out at 30°C for 24 h in supplemented MRS medium under the conditions listed in Table 1. Since the *Pediococcus* genus has limited breathing capacity and its multiplication is favored under microaerophilic conditions

Table 2. Optimization of *Pediococcus pentosaceus* growth with agitation in rotary shaker or without agitation at 30ºC for 24 h in MRS medium supplemented with nitrogen sources

MRS + supplements (%)	150 rpm			200 rpm			Anaerobic			Anaerobic 200 rpm		
	DM (g/L)	pH (I)	pH (F)	DM (g/L)	pH (I)	pH (F)	DM (g/L)	pH (I)	pH (F)	DM (g/L)	pH (I)	pH (F)
Pep 0.5	2.14	6.20	3.99	2.41	6.20	4.02	1.50	6.20	3.91	2.17	6.20	3.89
Pep 1.0	2.93	6.20	3.95	3.14	6.20	4.00	1.98	6.20	3.89	3.04	6.20	3.85
Pep 1.5	3.19	6.20	3.96	3.42	6.20	4.04	2.78	6.20	3.85	2.42	6.20	3.87
Ure 0.5	1.93	6.20	4.11	1.91	6.20	4.09	1.79	6.20	3.97	2.02	6.20	4.00
Ure 1.0	1.70	6.20	4,14	1.59	6.20	4.23	1.85	6.20	4.01	2.20	6.20	4.03
Ure 1.5	1.51	6.20	4.22	0.00	6.20	7.80	1.83	6.20	4.00	2.01	6.20	4.05
Control	1.00	6.20	4.13	2.05	6.20	4.05	0.92	6.20	4.33	2.00	6.20	4.33

Legend: DM: dry mass, Pep: peptone, Ure: urea, Control: MRS medium without nitrogen supplement, pH (I): initial pH, pH (F): final pH.

According Table 2, MRS medium supplemented with 1.5% peptone and under agitation of 200 rpm significantly improved *P. pentosaceus* growth, showing the highest cell mass concentration (3.42 g/L) after 24 h of cultivation.

It is noteworthy that, although the 200 rpm anaerobic conditions were not originally proposed in the experimental design (Table 1), it was suggested by the BLIS antimicrobial activity results against *L. sakei*, after its production in MRS medium without nitrogen supplementation and storage at 4ºC for 2 months (Figure 2). BLIS was in fact able to maintain its activity only when produced at 200 rpm under either anaerobiosis or microaerophilia.

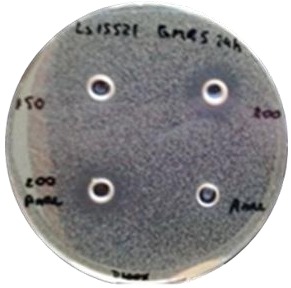

Figure 2. Antimicrobial activity against *Lactobacillus sakei* ATCC 15521 of BLIS produced by *Pediococcus pentosaceus* ATCC 43200 after 24 h of cultivation in MRS medium under different agitation conditions (rpm) and storage at 4ºC for 2 months. Up left: 150 rpm, up right: 200 rpm, down left: anaerobic 200 rpm, down right: anaerobic.

Figure 3 illustrates the kinetics of *P. pentosaceus* growth in MRS medium supplemented with 1.5% peptone under different agitation conditions performed in the bioreactor.

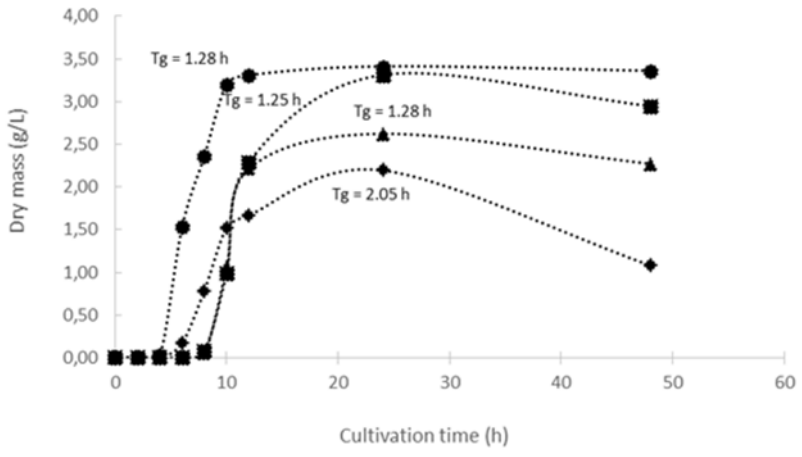

Figure 3. Growth curve (0-48 h) of *Pediococcus pentosaceus* ATCC 43200 cultivated at 30ºC in MRS medium added of 1.5% peptone (MRS-PEP) and without peptone pH 6.0 under the following aeration conditions: Control 200 rpm (◆), MRS-PEP anaerobiosis (without agitation) (▲), MRS-PEP microaerophilic (without agitation) (■), MRS-PEP anaerobiosis 200 rpm (●).

According to Table 3, the highest cell mass concentration (3.41 g/L) was obtained after 24 h of *Pediococcus* cultivation at 30ºC in MRS medium supplemented with 1.5% peptone performed in the bioreactor under anaerobiosis and agitation of 200 rpm. A coincident value (3.42 g/L) obtained when *P. pentosaceus* was grown in the same medium under agitation of 200 rpm for 24 h in rotatory shaker (Table 2) confirms the importance of agitation for its growth and its preference for anaerobiosis. Moreover, the generation time (T_g) was reduced by about 62% in the presence of 1.5% peptone when it was cultivated without agitation under anaerobiosis or microaerophilia (T_g = 1.25 h) or with agitation of 200 rpm under anaerobiosis (T_g = 1.28 h) compared with the control (T_g = 2.05 h) (Figure 3). Callewaert and De Vuyst (2000), studying the fed-batch growth of *Lactobacillus amylovorus* DCE 471 in modified MRS medium (40 g/L glucose) and medium with different nitrogen sources, reported a maximum dry cell concentration of 5.0 g/L and a maximum viable cell count of 1.9×10^9 per mL in the peptone-containing medium. These results are not so far from those obtained in the present work when *P. pentosaceus* was cultivated in MRS medium supplemented with peptone in bioreactor.

Figure 3 also shows that agitation of the culture medium was able to reduce the lag phase duration and, consequently, to accelerate the appearance of the exponential phase of growth. Under optimized culture conditions in bioreactor (Figure 3; Table 3), *P. pentosaceus* did in fact grow for 24 h, showing an exponential phase starting from 4 to 8 h, a stationary phase from 10 to 12 h and a decline one from 24 h.

As a conclusion, *P. pentosaceus* cultivated under microaerophilia and anaerobiosis without agitation (Figure 3) suffered a 4 h-delay in the appearance of the exponential growth phase compared with anaerobiosis in bioreactor under 200 rpm agitation (Table 3).

Table 3. Growth of *Pediococcus pentosaceus* (0-48 h) at 30ºC cultivated in MRS supplemented with 1.5% peptone in bioreactor under anaerobiosis and 200 rpm agitation, and BLIS antimicrobial activity against lactic acid bacteria

Cultivation time (h)	Dry mass (g/L)	CFU/mL	pH	Antimicrobial activity (mm)					
				L. plantarum		*C. piscicola*		*L. sakei*	
				OD 0.3	D100x	OD 0.3	D100x	OD 0.3	D100x
0	0.000	5.0×10^6	6.20	NA	NA	NA	NA	NA	NA
2	0.000	9.5×10^6	6.20	NA	NA	NA	NA	NA	NA
4	0.024	5.5×10^7	5.70	NA	NA	NA	NA	NA	NA
6	1.035	2.1×10^8	4.67	*	12.60	*	11.40	13.40	17.00
8	2.360	4.2×10^8	4.22	10.50	13.50	10.70	12.00	15.70	18.00
10	3.200	2.2×10^8	4.16	10.00	14.00	10.40	12.50	15.00	19.00
12	3.306	3.0×10^8	4.02	9.50	13.30	10.00	12.50	14.40	18.70
24	3.409	1.5×10^8	3.93	11.40	19.20	11.80	17.80	13.50	19.50
48	3.358	1.2×10^8	3.80	10.00	17.30	9.30	15.85	11.00	17.40

Legend: CFU/mL: colony-forming unit per milliliter, OD 0.3: optical density of 0.3, D100x: diluted 100 times from OD 0.3, *L. plantarum*: *Lactobacillus plantarum* CECT 221, *C. piscicola*: *Carnobacterium piscicola* CECT 4020, *L. sakei*: *Lactobacillus sakei* ATCC 15521, NA: no activity, *: weak activity and difficult definition of the inhibition halo.

3.2. Optimization of BLIS antimicrobial activity

As shown in Table 3, bacteriocin activity in bioreactor started at the beginning of the exponential growth phase (6-8 h), which means that the synthesis of this metabolite is growth associated. The same result was reported by Callewaert and Vuyst (2000) in their study on bacteriocin production by *L. amylovorus*, whose volumetric productivity was dependent on the total biomass formation (De Vuyst, & Vandamme, 1992; De Vuyst, Callewaert, & Crabbé, 1996; Moortvedt-Abildgaard et al., 1995; Callewaert, & De Vuyst, 2000).

The antimicrobial activity of BLIS produced in rotary shaker by *P. pentosaceus* cultivated in MRS medium supplemented or not with peptone or urea in different concentrations (0.5, 1.0 and 1.5%) is illustrated in Figure 4. One can see that such an activity was strongly strain specific, in that it was always satisfactory (diameter of the inhibition halo ≥ 10 mm) against *Staphylococcus aureus* CECT 239, *Lactobacillus plantarum* CECT 221, *Carnobacterium piscicola* CECT 4020 and *Lactobacillus sakei* ATCC 15521, regardless of the addition or not of any nitrogen source, of the type of nitrogen source and of its concentration, even though *P. pentosaceus* growth was significantly improved and its T_g reduced in the presence especially of peptone (Figure 3). On the other hand, it was poor (diameter of the inhibition halo < 10 mm) or even negligible against *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* CECT 116, *Listeria monocytogenes* CECT 934 and NADC 2045 and *Salmonella enterica* CECT 724 (Figure 4).

To investigate the antimicrobial activity of BLIS produced by *P. pentosaceus* in bioreactor, we selected the anaerobic conditions and medium composition previously optimized in shaken flasks (MRS medium supplemented with 1.5% peptone, 30ºC, pH 6.0, 200 rpm). To this purpose, *L. sakei* ATCC 15521, *L. plantarum* CECT 221 and *C. piscicola* CECT 4020 (Figure 5, panels A, B, C, respectively) were selected as indicator strains for exhibiting the highest inhibition halos in runs performed in rotary shaker.

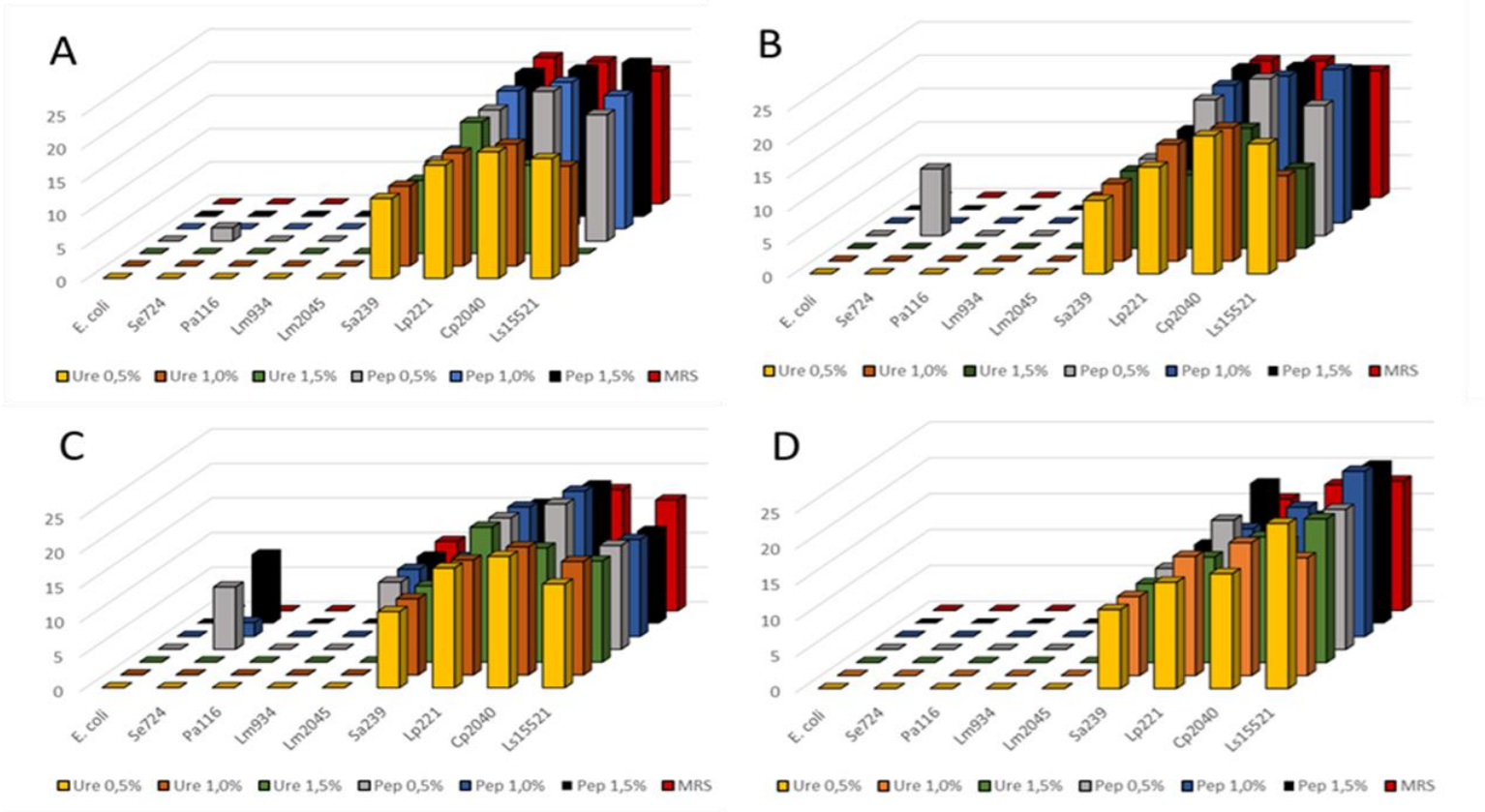

Figure 4. Antimicrobial activity of BLIS produced in rotary shaker by *Pediococcus pentosaceus* at 30°C after 24 h of cultivation in MRS without and with peptone or urea in different concentrations (0.5, 1.0 and 1.5%), pH 6.0 ± 0.2. The results are expressed as diameter of the inhibition halo (mm). Each panel represents different aeration and agitation conditions: A: rotary shaker under 150 rpm; B: rotary shaker under 200 rpm; C: anaerobic jar without agitation; D: rotary shaker (anaerobic jar) under 200 rpm. Legend: *E. coli*: *Escherichia coli* ATCC 25922; SE724: *Salmonella enterica* CECT 724; Pa116: *Pseudomonas aeruginosa* CECT 116; Lm934: *Listeria monocytogenes* CECT 934; Lm2045: *Listeria monocytogenes* NADC 2045; Sa239: *Staphylococcus aureus* CECT 239; Lp221: *Lactobacillus plantarum* CECT 221; Cp4020: *Carnobacterium piscicola* CECT 4020; Ls15521: *Lactobacillus sakei* ATCC 15521.

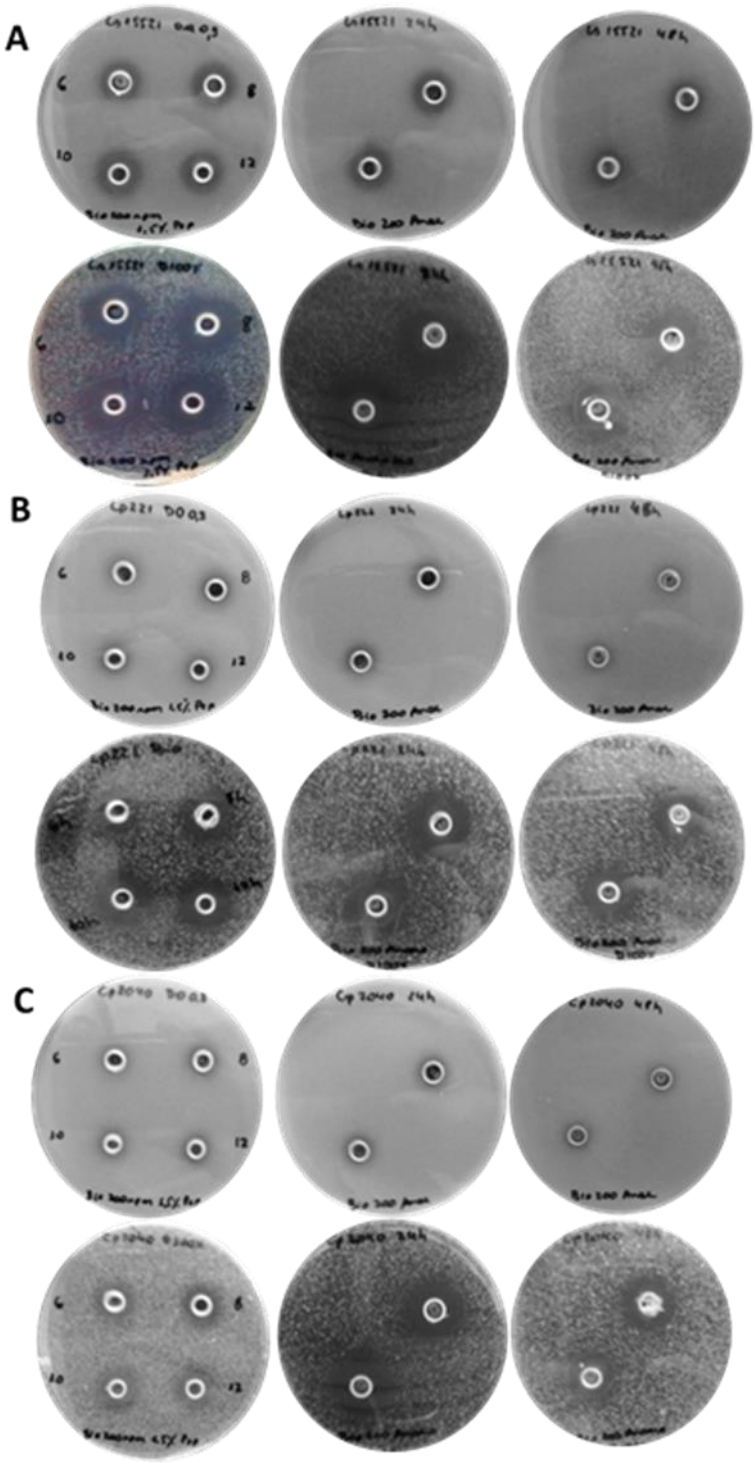

Figure 5. Antimicrobial activity of BLIS produced in bioreactor by *Pediococcus pentosaceus* in MRS medium supplemented with 1.5% peptone and tested against lactic acid bacteria suspensions of OD 0.3 (upper lines, panels A, B, C) and 100 times diluted from OD 0.3 (downer lines, panels A, B, C). (A) *Lactobacillus sakei* ATCC 15521, (B) *Lactobacillus plantarum* CECT 221, (C) *Carnobacterium piscicola* CECT 4020. From the left to the right: BLIS solution withdrawn in 6, 8, 10 and 12 h (1stcolumn); 24 h (2nd column) and 48 h (3rd column).

As shown in Table 3, the inhibition halos were expressive, after both 10 and 24 h of cultivation, against all the three indicator strains used (*L. plantarum* = 14.00, 19.20 mm; *C. piscicola* = 12.50, 17.80 mm and *L. sakei* = 19.00, 19.50 mm, respectively), with a special sensitivity of *L. sakei*, but regardless of cell concentration (OD = 0.3, corresponding to 6-8 × 10^6 CFU/mL, or x100 time diluted). Callawaert and De Vuyst (2000) observed the same profile of bacteriocin activity, which started at the beginning of the exponential growth phase, increased and reached its maximum value in the stationary growth phase, i.e. under conditions of glucose depletion.

Such inhibition halos were quite large when compared with literature; therefore, *P. pentosaceus* BLIS can be considered a promising antimicrobial compound.

3.3.SDS-PAGE and 16S rDNA gene sequencing

Submission of BLIS-containing broth without any previous purification to SDS-PAGE evidenced the presence of a band corresponding to a molecular weight of about 75-80 kDa (Figure 6). This molecular weight agrees with that reported (80 kDa) by Piva and Headon (1994) for Pediocin A produced by the same *P. pentosaceus* strain, which was classified as a bacteriocin belonging to class III.

16S rDNA Sequencing was then used to confirm the genus and species of BLIS-producing microorganism. Comparison of gene sequence with those of the Pubmed (Blast) database evidenced 98% homology with *P. pentosaceus*, thereby confirming our previous expectations.

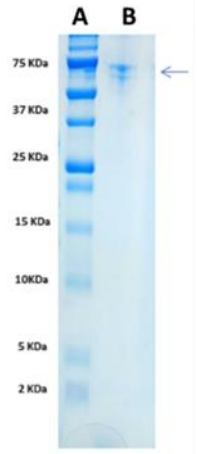

Figure 6. Polyacrylamide gel electrophoresis of BLIS without purification. A: molecular weight marker, B: BLIS-containing fermented broth.

3.4. Determination of BLIS MIC_{50}

Many researchers use the agar-diffusion method to evaluate the inhibitory effect of antimicrobials; however, this method sometimes provides inaccurate results due to the variability of agar-medium components (Azevedo, Molinari, & Oliveira, 2018), the antimicrobial ability to diffuse through the agar gel, and the possible presence of detergents (e.g. Tween) to improve diffusion. Moreover, in some cases, the inhibition halo may be undetectable, which does not necessarily mean absence of antimicrobial activity. Many researchers have then been using turbidimetry as an option to determine the minimal inhibitory concentration to reduce bacterial growth by 50% (MIC_{50}) (Cabo et al., 1999). For instance, BLIS did not seem to have any activity against *L. innocua* NCTC 11288 (Fig. 7A) and only a weak one against *L. seeligeri* NCTC 11289 (Fig. 7B) according to the former method, whereas both strains proved to be sensitive according to the latter.

Both broths without any dilution or 1x diluted did in fact display 100% inhibition of *L. innocua* growth, the 5x diluted one only 50% inhibition and the more diluted ones no inhibition (Fig. 8A). On the other hand, *L. seeligeri* growth was inhibited by 50% by the broth as such, 100% by 1x and 5x diluted broths, 80% by the 10x diluted one, while no inhibition was exerted by broths even more diluted (Fig. 8B).

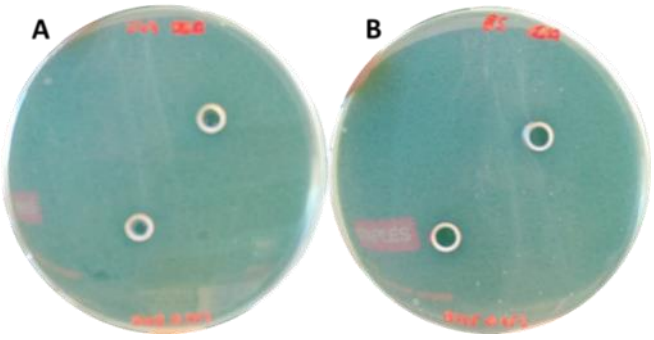

Figure 7. Antimicrobial activity against *Listeria innocua* NCTC 11288 (A) and *Listeria seeligeri* NCTC 11289 (B) of BLIS produced by *Pediococcus pentosaceus* after 10 h of anaerobic cultivation at 30°C. Cell concentration of 3×10^6 CFU/mL.

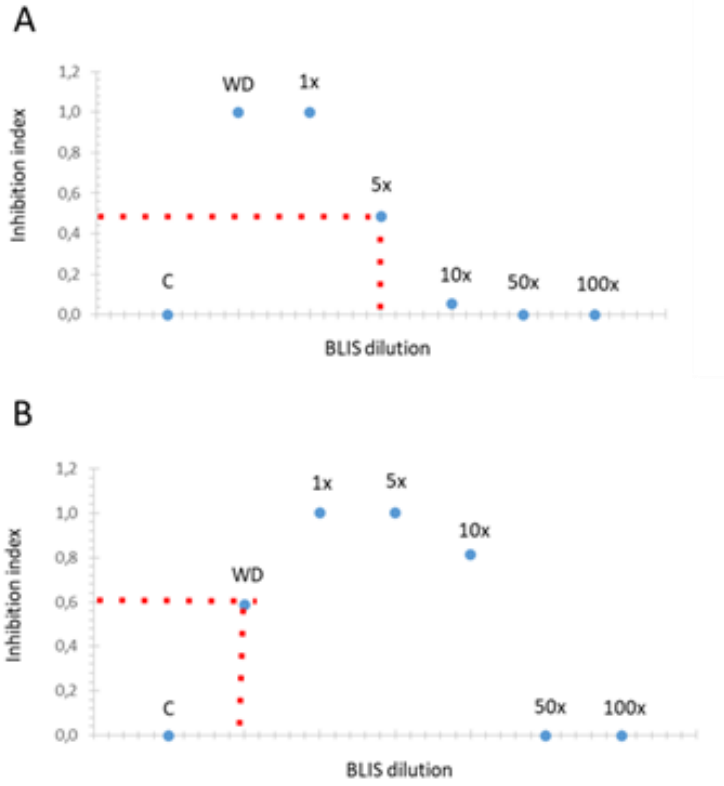

Figure 8. BLIS minimal inhibitory concentration (MIC_{50}) to reduce *Listeria innocua* NCTC 11288 (A) and *Listeria seeligeri* NCTC 11289 (B) growth by 50%. Inhibition index of 1.0 indicates 100% inhibition of growth. C = control, WD = without dilution.

4. Conclusion

In order to increase the antimicrobial activity of bacteriocin-like inhibitory substance (BLIS) produced and secreted by *Pediococcus pentosaceus* ATCC 43200, 1.5% peptone was supplemented to Man, Rogosa and Sharpe medium as an additional nitrogen source. Such an ingredient was able to accelerate the growth of this microorganism, shortened the occurrence of its exponential growth phase, reduced its generation time and improved BLIS antimicrobial activity either in flasks or in bioreactor, under either anaerobic or microaerophilic conditions. These results as a whole highlight the need of further research efforts in this direction as well as the potential of such a BLIS as an antimicrobial in food applications.

CHAPTER V[1]

Bacteriocin-like inhibitory substance of *Pediococcus pentosaceus*: quality parameters and effectiveness as biopreservative for control of *Listeria* in ready-to-eat pork ham

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ABSTRACT

The growing demand from consumers for foods free of synthetic chemicals has increased the search for natural food preservatives such as bacteriocin and bacteriocin-like inhibitory substance (BLIS) with adequate microbiological safety, sensory characteristics shelf life. BLIS produced by *Pediococcus pentosaceus* ATCC 43200 was effective in eliminating the growth of *Listeria seeligeri* NCTC 11289 (Lse) in ready-to-eat pork ham after 2 days (from 1.74 to 0.00 log CFU/g), suggesting its possible bactericidal effect during the first days of contact between BLIS and bacteria. BLIS showed its effectiveness by maintaining low Lse multiplication (3.57 log CFU/g), lower weight loss (2.7%) and low lipid peroxidation (0.63 mg MDA/Kg) compared with control (3.87 log CFU/g; 3.0%; 1.25 mg MDA/Kg). At the same time, the coloration of ham samples was maintained more intense according to the parameters luminosity, redness and yellowness. These results indicating the potential use of this BLIS as a biopreservative in the meat and other food industries.

Keywords: *Pediococcus pentosaceus*, Lactic acid bacteria, BLIS, Nisin, shelf life, Biopreservative

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1. Introduction

Consumer lifestyle has changed in the last decades and the growing demand from consumers for foods without chemical additives (Cortés-Zavaleta et al., 2014) and restrictions by regulations have created challenges for food manufactures in the searching of food natural conservatives. In addition to microbiological safety, products should have adequate sensory characteristics and longer shelf life (Nielsen & Rios, 2000, Moreira et al., 2005) and, in this way, natural ingredient has been increasingly considered as a suitable alternative, because of their proven antimicrobial action against food spoilage and foodborne pathogens. Contamination of food by pathogenic bacteria such as *Clostridium botulinum*, *Staphylococcus aureus*, *Bacillus cereus* and *Listeria monocytogenes* is still a problem for the food industry, with relevant morbidity rates even in developed countries (EFSA & ECDC, 2015). Therefore, there is a need to develop better conservation alternatives to improve the existing food technologies, making available to the population a better food quality and safer from the point of view of microbiological security (Schulz et al., 2003). Regarding food safety, spoilage bacteria concentration should not exceed 100 CFU/g to be considered as a minor risk for the population. In this way, the European Union's microbiological criterion for *Listeria monocytogenes* is define as ≤ 100 CFU/g for ready-to-eat products available on the market (EFSA & ECDC, 2014). According to Astiz (2008), besides the quality of the meat from a sanitary point of view, other parameters such as appearance and odor also influence the choice of meat (or meat-based products) to be consumed. The microorganisms in general use the food as a source of nutrients for their growth and this action can promote significant changes in the characteristics of the food, including taste, color, odor and texture, and the consumer will certainly reject it (Frazier & Westhoff, 1993) as soon as it is considered as a deteriorated food. It should be noteworthy that the difference between a deteriorated food and a contaminated food, in this last one, means the presence of pathogens responsible for causing illness in consumers (Franco, 1996). This means, contaminated food not necessarily causes illness to consumers, but alters the organoleptic properties of food of animal origin (e.g. cheese, ham).

According the data from the United States Department of Agriculture (USDA, 2017), pork meat is the most produced in the world and the global production is forecast up nearly 2% in 2018 to 113.1 million tons. Brazil occupies the third place in the world ranking of pork meat production with 3.7 million tons/year, with China and European

Union with higher production. In the world ranking of consumption, Brazil occupies the fourth position with 2.9 million tons/year, behind only China, European Union and Russia.

Due to the versatility of the use of pork meat for human food, whether in the preparation of cuts *in natura* or in the manufacture of a large number of sausages, salted and smoked, this should guarantee over the next years its world consumption leadership in relation to meat of other species (Fávero, 2002). For Brazil, there is a growth prospect of pork meat production up to 2023, with an annual growth rate of 1.9% (MAPA, 2013). However, the main factor that still limits the consumption of pork in Brazil is related to health issues, i.e. the high deterioration of processed pork due to pathogens.

A quite old but innovative technology of food biopreservation is the use of lactic acid bacteria (LAB) and their metabolites. LAB strains are recognized by *U.S. Food and Drug Administration* (FDA) as *Generally Regarded as Safe* (GRAS). LAB strains produce high value-molecules during the fermentation process. In fact, molecules with antimicrobial effects with bioconservative characteristics confer to the product increasing shelf life and microbiological safety. Furthermore, LAB metabolites have minimal impact on the nutrition and sensory properties of processed food products (García et al., 2010) and they are safe for human consumption (Giraffa, Chanishville & Widyastuti, 2010).

Certain LAB strains synthesize and secrete antimicrobial peptides called bacteriocins and also secondary metabolites able to inhibit the growth of competitive bacterial strains (Drider et al., 2006; Kjos et al., 2010; Hwanhlem et al., 2013), protozoa, fungi and viruses (Reddy et al., 2004). Bacteriocins are amphiphilic molecules with broad spectrum of action, different molecular structures and weights, displaying stability at high pH and thermal conditions (Nes et al., 1996; Zacharof & Lovitt, 2012; Kassaa et al., 2015). However, when this bacteriocin has not been completely characterized regarding its amino acid and the nucleotide sequences of the corresponding gene, the qualified term bacteriocin-like inhibitory substance (BLIS) is recommended (Tagg, Dajani, & Wannamaker, 1976; Jack, & Ray, 1995). We hypothesize that *Pediococcus*' BLIS may be an important new food preservative. Therefore, the aim of this study was to evaluate the effectiveness of bacteriocin-like inhibitory substance (BLIS) as biopreservative for control of *Listeria seeligeri* NCTC 11289 in ready-to-eat pork ham. The BLIS is produced and secreted by *Pediococcus pentosaceus* ATCC 43200 after fermentation of glucose-based Man, Rogosa and Sharp (MRS) medium, regarding quality parameters.

2. Material and Methods

2.1. Microbial cultures

For the development of the fermentative process, *P. pentosaceus* ATCC 43200 was used. This strain is also known as FBB61, and is isolated from fermented cucumber (Costilow et al., 1956). As a microorganism indicative of antimicrobial activity and as a contaminant of ready-to-eat pork ham, *L. seeligeri* NCTC 11289, kindly provided by Dr. Ciolacu from Department for Farm Animals and Veterinary Public Health of the University of Veterinary Medicine (Vienna, Austria), was used. Both microorganisms were grown at 37ºC overnight in appropriate culture medium (MRS Roth®, Karlsruhe, Germany) for *P. pentosaceus* and Brain Heart Infusion (BHI Roth®, Karlsruhe, Germany) for *L. seeligeri*. Both culture media were autoclaved (Tuttnauer 2540 ELV) at 121ºC for 12 min. The strains were cryopreserved and stored at -70ºC after addition of 20% (v/v) of glycerol to each medium.

2.2. Culture condition to obtain the BLIS

To prepare the culture, 1 mL of cryopreserved *P. pentosaceus* was transferred to a 500 mL Erlenmeyer flask containing 300 mL of MRS medium and incubated at 30ºC for 10 h without agitation.

2.3. Nisin solution

Nisin, a bacteriocin produced by *Lactococcus lactis* subsp. *lactis*, was obtained from DuPont Danisco Company, which has a trade name of Nisaplin®. Nisaplin is a heat-stable, non-dairy based antimicrobial that inhibits the majority growth of Gram-positive bacteria and can be used in food application, including dairy, culinary, meat, beverage and bakery products. Nisaplin solution (1%) was prepared in sterile distilled water and filtered through a membrane with 0.22 µm porosity (Millipore, Billerica, MA, USA) before use. It is noteworthy that the antimicrobial/biopreservative effect of BLIS from *P. pentosaceus* ATCC 43200 was compared to the same effect of Nisaplin.

2.4. Determination of BLIS antimicrobial activity

To detected the antimicrobial activity, BLIS production from *P. pentosaceus* ATCC 43200 was recovered by centrifugation at 4,470 ×*g* at 4°C for 15 min and the pH was adjusted to 6.0-6.5 by addition of NaOH (1N), heated to 70°C for 25 min to inactivate proteases and filtered through the membrane with 0.22 µm porosity (Millipore, Billerica, MA, USA). BLIS was tested against five indicator strains (*Lactobacillus sakei* ATCC 15521 (Ls), *Listeria seeligeri* NCTC 11289 (Lse), *Listeria innocua* NCTC 11288 (Li), *Enterococcus* spp., strains En2052 and En2865) and the test was performed by the agar well diffusion method. To performed this method, the overnight culture of the bioindicator strain was diluted with medium (MRS for *Ls* and BHI for the other strains) to give an optical density (OD) at 600 nm (Hitachi U-5100) of 0.3 which corresponding to 8.5×10^6 CFU/mL for Ls, 3.0×10^6 CFU/mL for Lse, 5.0×10^6 CFU/mL for *Li*, 3.0×10^6 CFU/mL for En2052 and 2.0×10^6 CFU/mL for En2865, then 150 µL of this suspension (OD 0.3) was added to 15 mL of melted soft agar MRS or BHI (0.75% w/v) and poured into Petri dishes. Fifty µL of denatured supernatant was added into each well. All plates were incubated for 16-18 h at 37°C in duplicate. The antagonistic activity in arbitrary unit/mL (AU/mL) was calculated (Bhaskar et al., 2007; Azevedo et al., 2017) as a measure of BLIS production.

2.5. BLIS biopreservative efficiency in ready-to-eat sliced pork ham

To investigate the efficiency of BLIS against the growth of the bioindicator strain *L. seeligeri*, ready-to-eat pork ham slices were purchased in a local supermarket in Vienna (Austria) and transported in iced thermal box to the laboratory for analysis within 5 minutes. The pork ham slices were aseptically cut into pieces of 25 g and, to reduce the possible contaminants, the pieces were exposed to UV light for 30 min on each surface. After this process, the pieces were artificially contaminated by spraying with 500 µL of a culture suspension (OD 0.3) of Lse, on each surface.

Followed the artificial contamination, the samples were divided into four groups: control (Lse contamination without antimicrobial treatment), BLIS (Lse contamination + BLIS treatment), NISA (Lse contamination + Nisaplin 1% treatment) and COMB (Lse contamination + BLIS + Nisaplin 1% (50%/50%) treatment). The antimicrobial treatment comprised the application by sprayed of 500 µL of BLIS and/or Nisaplin 1% on each

surface of pork ham pieces. Uniform spraying of the surface of pork ham pieces was achieved using a spray gum. Immediately after this process, samples were vacuum-packaged (Komet Germany Vacuboy) in appropriate plastic packaging (Siegelrandbeutel 180 x 225) and weighted. Samples were stored at 4^{o}C and the shelf life days evaluated were: 0 (d0), 2 (d2), 6 (d6) and 10 (d10).

2.6. Microbiological analysis

To monitor the growth of Lse during the storage, 225 mL of 0.3% sterile saline was added to the plastic bags containing the pork ham pieces and homogenized for 2 min in a Stomacher (Lab-Blender 400) and, the resulting suspension, was serially diluted 10-fold prepared by diluting 1 mL in 9.0 mL of 0.3% sterile saline. Ten microliters of each dilution was deposited on the surface of selective medium (10 mL) for *Listeria* (Oxford Listeria Agar - Roth®, Karlsruhe, Germany) after its solidification and the plates were incubated at 37^{o}C for 24 h. All *Listeria* species hydrolyze esculin, producing 6,7-dihydroxycoumarin which reacts with ferric ions producing blackening of the medium. All bacteria counts (CFU/g) recovered from ready-to-eat pieces pork ham were converted to logarithms before computing their means and standard deviations. Bacterial counts were determined in duplicate and all of the experiments described here were independently performed three times.

2.7. Weight loss

To determine the weight loss of the samples (control and treated), the packages containing pieces of pork ham were weighed in a semi-analytical balance and the weight loss during the storage was determined by the equation below, being m_i the mass at the initial storage time (d0) and m_f the mass at the later day of the storage.

$$WL\ (\%) = \frac{(m_i - m_f) \times 100}{m_i}$$

2.8. pH measurements

The pork ham pH was measured after grinding (Home Turbo Deluxe Mixer) the samples at each predetermined shelf life time (d0, d2, d6, d10), using a pH meter (Testo 204). Two readings were obtained for each sample.

2.9. Lipid oxidation

Lipid oxidation of ready-to-eat pork ham samples was assessed in duplicate by the 2-thiobarbituric acid (TBA) method. Five grams of each pork ham sample was homogenized with 25 mL of 7.5% (w/v) trichloroacetic acid using a mixer (Home Turbo Deluxe Mixer). The homogenate was decanted through a paper filter (MN 619 EH ¼ Ø 185 mm, Macherey-Nagel). Five milliliters of the filtrate was mixed with 5.0 mL of the TBA reagent (0.02 mol/L) and this mixture was heated in a heater block (Dri-Block Heater DB-3D, model FDB03DP, Techne) at 100ºC for 40 min. After the mixture had cooled to room temperature, the absorbance was measured at 532 nm and thiobarbituric acid reactive substances (TBARS) values were calculated from a standard curve of malonaldehyde (MDA) (R^2=0.9956) using 1,1,3,3,-tetraethoxipropane (TEP) as standard, and expressed as mg MDA/Kg of each sample.

2.10. Color determination

Color stability of ready-to-eat pork ham samples was determined as a parameter of quality to assess how color changes when natural antimicrobials were used as food biopreservative. Subjective percent discoloration (photometric image) was determined on samples in vacuum packages using a spectrophotometer CM-600d (Konica Minolta, Osaka, Japan) with an 8 mm diameter measurement area, illuminant D65, operate in the CIELAB system. The L* value referred to color lightness, ranging from 0 (black) to 100 (white); the a* value related to the span of red-green color, ranging from -100 (greenness) to +100 (redness); and b* value indicated the extent of yellow-blue color, ranging from -100 (blueness) to +100 (yellowness) (Elias, 1993). Values for L* (brightness), a* (redness), and b* (blue to yellow) were recorded in three different parts of the samples.

From the parameters a* and b*, the chromatographic coordinate such as saturation indices (C*) and the hue angle (h*) were calculated by the following formulas (MacDougal, 1994; Ramos & Gomide, 2007):

$$C^* = (a^{*2} + b^{*2})^{1/2}$$

$$h^* = \tan^{-1}(b^*/a^*)$$

2.11. Statistical analysis

The experimental data were presented as mean values. Variations with respect to the mean values were presented as standard deviations. Mean values of concentrations were submitted to analysis of variance (ANOVA) by the Statistica Software 13.3 (TIBICO Software Inc., USA). They were compared using the Tukey's post-hoc test and considered significantly different at $p < 0.05$.

3. Results and Discussion

3.1. BLIS antimicrobial activity

The term BLIS is recommended to use when a newly discovered bacteriocin is not completely characterized regarding its amino acid sequence and the nucleotide sequence of the corresponding structural gene (Jack, Tagg, & Ray, 1995; Tagg, Dajani, &Wannamaker, 1976) then, for this reason, the antimicrobial produced by *P. pentosaceus* was determined as BLIS. Its antimicrobial activity was compared to nisin (Nisaplin®), the most commonly natural antimicrobial compound used and the only accepted antimicrobial substance to be used in pure form in a wide range of foods (Settanni & Corsetti, 2008; Khajehali et al., 2011).

The first commercial preparation of nisin (Nisaplin®) was made in 1953, from the fermentation of skimmed milk by strains of *Lactococcus lactis*, by Aplin and Barret, Ltd., England, and currently marketed by Danisco A/S, Denmark (Ross & Hill, 2001; Maragkoudakis et al., 2009; Tafreshi et al., 2010; Basto, Coutinho, & Coelho, 2010). In 1988, FDA approved its use in food, after its recognition as GRAS. Each gram of Nisaplin® contains approximately 2.5% of nisin, which corresponds to 10^6 IU (International Units), i.e. 1 µg of pure nisin equals 40 IU. Nisaplin® is an extremely stable

product, does not reduce its activity in up to 2 years when stored in a dry, light-free environment and at temperatures below 25ºC (Delves-Broughton, 2005; JAY, 2005).

The antimicrobial activity of BLIS, Nisaplin and both antimicrobial in combination (Figure 1), was evaluated against *Lactobacillus sakei* ATCC 15521 (Ls) strain. *L. sakei* is a specie of lactic acid bacterium commonly isolated and identified as a deteriorating product of vacuum-packed fresh meat products (Castellano, Holzapfel, Vignolo, 2004). It is a close related strain to the nisin producer (*Lactococcus lactis* subsp. *lactis*). In the present study, Ls was more sensitive to BLIS activity, showing diameters of inhibition halos of 14.50 mm at Ls OD 0.3 and 18.00 mm at Ls diluted 100x from OD 0.3 ($p < 0.05$). Even though Ls is a closely related bacteria to the nisin producer, its sensitivity to this bacteriocin was around 33% lower (8.75 mm) than BLIS when Ls OD 0.3 was diluted 100x (Table 1). The results expressed in AU/mL are also presented in Table 1, which corresponds to the results expressed in diameters of inhibition halos (mm).

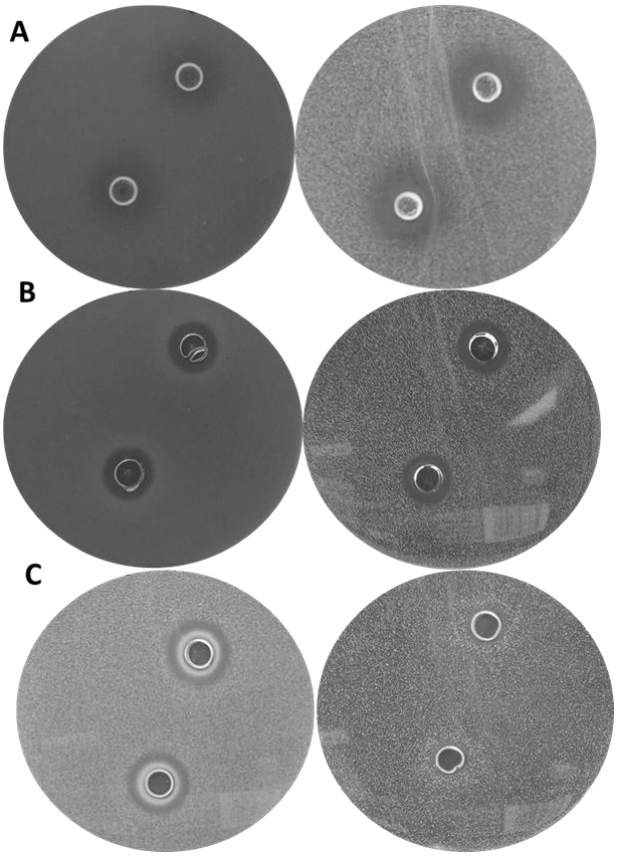

Figure 1. Antimicrobial activity of BLIS (A), Nisaplin 1% (B) and BLIS + Nisaplin 1% (C) against the indicator strain *Lactobacillus sakei* ATCC 15521. From left to the right: indicator strain at OD 0.3 and indicator strain diluted 100x from OD 0.3.

Table 1. Antimicrobial activity[a], expressed in diameter of inhibition halo (mm) and critical dilution rate expressed as arbitrary units per mL (AU/mL) of BLIS produced by *P. pentosaceus* ATCC 43200 after 10 h of fermentation, Nisaplin® (1%) and the combination of both antimicrobials

Bioindicator strains		Antimicrobials substances					
		BLIS		NISA		COMB	
		OD 0.3	Dil 100 x	OD 0.3	Dil 100 x	OD 0.3	Dil 100 x
Halo (mm)	Ls	14.50 ± 0.00^{X}	$18.00 \pm 0.00^{A,c}$	12.65 ± 0.07^{Y}	$12.00 \pm 0.42^{B,a}$	15.50 ± 0.71^{X}	8.75 ± 0.35^{C}
	En2052	-	16.20 ± 0.28^{b}	-	12.50 ± 0.00^{a}	-	*
	En2865	-	16.45 ± 0.21^{b}	-	12.30 ± 0.00^{a}	-	*
	Lse	-	15.50 ± 0.21^{b}	-	12.50 ± 0.00^{a}	-	*
	Li	-	9.50 ± 0.00^{c}	-	9.70 ± 0.00^{c}	-	*
AU/mL	Ls	290.00 ± 0.00^{X}	$360.00 \pm 0.00^{A,c}$	253.00 ± 0.11^{Y}	$240.00 \pm 0.49^{B,a}$	310.00 ± 0.14^{X}	175.00 ± 0.70^{C}
	En2052	-	324.00 ± 0.56^{b}	-	250.00 ± 0.00^{a}	-	*
	En2865	-	329.00 ± 0.42^{b}	-	246.00 ± 0.00^{a}	-	*
	Lse	-	310.00 ± 0.14^{b}	-	250.00 ± 0.00^{a}	-	*
	Li	-	190.00 ± 0.00^{c}	-	194.00 ± 0.00^{c}	-	*

Legend: BLIS = bacteriocin-like inhibitory substance, NISA = Nisaplin®, COMB = combination of BLIS and NISA (50%/50%), OD 0.3 = optical density of 0.3, Dil 100x = Dilution of 100 x from OD 0.3, Ls = *Lactobacillus sakei* ATCC 15521, En2052 = *Enterococcus* 2052, En2865 = *Enterococcus* 2865, Lse = *Listeria seeligeri* NCTC11289, Li = *Listeria innocua* NCTC 11288, - = not evaluated, * = difficult to measure (small halo). [a] Values are the mean of duplicates plus its standard deviation. Different letters in the same column mean statistically significant difference among the values of the same parameter, according to the test of Tukey ($p < 0.05$). Letters that belongs to the same statistical group: X, Y; A, B, C; a, b, c.

BLIS and Nisaplin presented efficient activity against Ls (Table 1). However, BLIS showed greater inhibitory activity (OD = 290.00 AU/mL; Diluted 100x = 360.00 AU/mL) than Nisaplin (OD = 253.00 AU/mL; Diluted 100x = 240.00 AU/mL) regardless of the concentration of the indicator strain ($p < 0.05$). The synergism between BLIS and Nisaplin (175 AU/mL) (Figure 1 C; Table 1) was not so effective because the isolated BLIS presented greater activity (360 AU/mL) when it was tested against Ls diluted 100x from OD 0.3. Nevertheless, this antimicrobial combination demonstrated greater efficiency when evaluated against Ls in the highest concentration (OD 0.3) (BLIS = 290.00 AU/mL; BLIS + NISA = 310.00 AU/mL).

The visual difference of the inhibition halo presented when antimicrobials were combined should be highlighted. The inhibition halo was a double circle (Fig. 1 C, on left) and not a single one, as observed in BLIS and Nisaplin (Figure 1 A, B, on left). The interaction of components of both antimicrobials and the agar medium promoted somehow a different way of interaction and/or agar diffusion of these molecules together. This was also possibly influenced by the pH, temperature and matrix of the agar medium, as already described by Azevedo, Molinari, and Oliveira (2018).

The antimicrobial activity of BLIS against *Enterococcus* strains and Lse was also higher than nisin activity (Figure 2, Table 1) except for *Li*, whose inhibition was the same for both antimicrobials. It is noteworthy that BLIS presented higher antimicrobial activity than Nisaplin, although this new molecule is in its crude state and its activity may be improved after its total or partial purification.

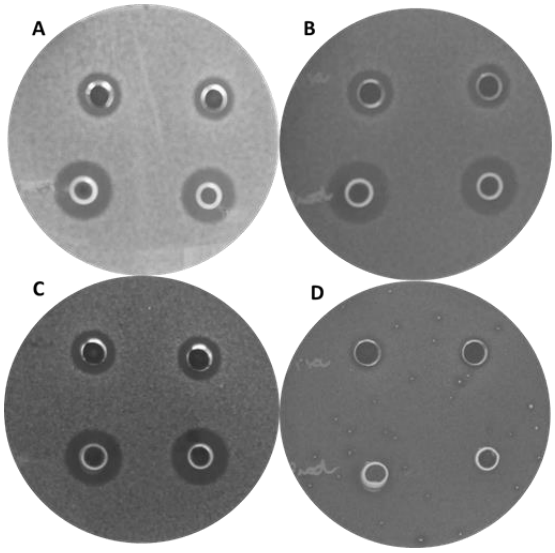

Figure 2. Antimicrobial activity of BLIS (lower wells) and Nisaplin 1% (upper wells) against bioindicator strains diluted 100x from OD 0.3. (A): *Enterococcus* sp. 2052 (En2052); (B): *Enterococcus* sp. 2865 (En2865); (C): *Listeria seeligeri* NCTC 11289 (Lse); (D): *Listeria innocua* NCTC 11288 (Li).

3.2. Biopreservative of ready-to-eat pork ham

The growth of the bioindicator strain Lse on the ready-to-eat pork ham was followed 10 days, and the treatments with the antimicrobial produced by *P. pentosaceus* (BLIS), Nisaplin® (NISA) and both antimicrobials in combination (COMB) were compared with the control (sample without addition of any preservative substance).

The effect of the treatments on the survival of the Lse strain, followed for 10 days (d0, d2, d6 and d10), is shown in Table 2. The average Lse population recovered from pork ham slices after inoculation (control) was 1.85 ± 0.26 log CFU/g and its growth was considerable (3.87 ± 0.24 log CFU/g) and significant ($p < 0.05$) after 10 days under refrigeration (4.0 ± 0.5°C), i.e. the bacteria population increased by 2 log CFU/g in the control. BLIS treatment was completely effective in eliminating the growth of Lse after 2 days (from 1.74 ± 0.24 log CFU/g to 0.00 ± 0.00 log CFU/g), which suggests its possible bactericidal effect on the first days of contact (d0 and d2) between BLIS and bacteria. Lse was able to grow after d6 and d10 however, its growth was significantly lower in BLIS-treated samples (1.80 ± 0.25 log CFU/g and 3.57 ± 0.04 log CFU/g, respectively) than the Lse growth in the control samples (3.69 ± 0.12 log CFU/g and 3.87 ± 0.24 log CFU/g) ($p < 0.05$).

The different activity profile between BLIS and Nisaplin is shown in Table 2. On the first contact (d0) until the second day (d2), Nisaplin was not efficient in preventing the growth of Lse, i.e. on d2; Lse growth was coincident between Nisaplin and control (1.85 ± 0.26 log CFU/g and 1.74 ± 0.24 log CFU/g, respectively). A possible explanation for this behavior may be related to the longer diffusion time of Nisaplin in the ham matrix. The desorption of nisin seems to be favored by the storage time of the product, which may justify the increase of the antimicrobial activity of nisin along to the shelf life (Mori, 2013). After d6, the efficiency of Nisaplin in preventing any growth of Lse was significant and remained even at d10 (d6 and d10 = 0.00 ± 0.00 log CFU/g) ($p < 0.05$), which was not the case for BLIS, COMB and control treatments.

Table 2 Growth of *Listeria seeligeri* NCTC11289, pH, weight loss and TBARS index in artificially contaminated ready-to-eat pork ham in vacuum-package storage at 4°C and treated with antimicrobials substances during shelf life of 10 days

Parameters	Days	Control	BLIS	NISA	COMB
log CFU/g	0	$1.85 \pm 0.26^{b,c,d}$	1.74 ± 0.24^{b}	$1.92 \pm 0.27^{c,d}$	$1.89 \pm 0.26^{c,d}$
	2	1.74 ± 0.24^{b}	0.00 ± 0.00^{a}	$1.85 \pm 0.26^{b,c,d}$	0.00 ± 0.00^{a}
	6	$3.69 \pm 0.12^{b,c,d}$	$1.80 \pm 0.25^{b,c,d}$	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	10	3.87 ± 0.24^{d}	$3.57 \pm 0.04^{b,c}$	0.00 ± 0.00^{a}	$1.89 \pm 0.26^{c,d}$
WL (%)	10	3.00 ± 0.12^{b}	2.77 ± 0.23^{a}	3.10 ± 0.21^{c}	3.20 ± 0.13^{d}
pH	0	$6.12 \pm 0.18^{b,c,d}$	5.90 ± 0.02^{a}	$6.16 \pm 0.05^{c,d}$	$6.12 \pm 0.06^{b,c,d}$
	2	6.20 ± 0.01^{d}	$5.86 \pm 0.00^{a,b,c,d}$	$6.15 \pm 0.02^{b,c,d}$	$6.17 \pm 0.01^{c,d}$
	6	$6.04 \pm 0.01^{b,c,d}$	$5.81 \pm 0.04^{a,b,c}$	$6.14 \pm 0.02^{b,c,d}$	$6.08 \pm 0.06^{b,c,d}$
	10	$6.11 \pm 0.00^{b,c,d}$	$5.80 \pm 0.01^{a,b}$	$6.11 \pm 0.04^{b,c,d}$	$6.03 \pm 0.04^{b,c,d}$
TBARS (mg MDA/Kg)	0	0.18 ± 0.00^{c}	0.14 ± 0.00^{b}	0.09 ± 0.00^{a}	0.17 ± 0.00^{c}
	2	0.33 ± 0.00^{d}	0.55 ± 0.00^{i}	0.47 ± 0.00^{h}	0.67 ± 0.01^{l}
	6	0.44 ± 0.00^{f}	0.43 ± 0.01^{e}	$0.46 \pm 0.00^{g,h}$	$0.45 \pm 0.00^{f,g}$
	10	1.25 ± 0.01^{m}	0.63 ± 0.00^{k}	0.66 ± 0.00^{l}	0.60 ± 0.00^{j}

Legend: CFU = colony forming unit, WL = weight loss, TBARS = thiobarbituric acid reactive substances, BLIS = bacteriocin-like inhibitory substance, NISA = Nisaplin®, COMB = combination of BLIS and NISA (50%/50%). Values are the mean of duplicates plus its standard deviation). Different letters mean statistically significant difference among the values of the same parameter, according to the test of Tukey ($p < 0.05$).

The growth of Lse in the combination of both antimicrobials (COMB) was quite consistent with the results obtained in the individual's treatments since the Lse growth pattern remained the same. Initially (d0) the growth in COMB was the same as in the control (1.89 ± 0.26 log CFU/g and 1.85 ± 0.26 log CFU/g ($p > 0.05$), respectively), probably due to the presence of Nisaplin. On d2, any bacteria growth was the same as observed in BLIS treatment, possibly result obtained because of the presence of BLIS and on d6 there was also any growth in consequence of the presence of Nisaplin. On d10, the bacteria growth was coincident with d0 (1.89 ± 0.26 log CFU/g) however, this growth was significantly lower than in the control (3.87 ± 0.24 log CFU/g) and in the BLIS (3.57 ± 0.04 log CFU/g), showing a positive effect (synergistic effect) of the combination when both antimicrobials was applied directly to food (ready-to-eat pork ham). Only Nisaplin (NISA treatment) was able to inhibit completely growth of Lse after the d6. In this way, it is possible to suggest that no bacteria growth in COMB treatment on d10 was because of the presence of Nisaplin.

Even the synergistic effect of BLIS and NISA did not seem to be as efficient as the size of the inhibition halo when evaluated by the agar diffusion method (Table 1) in the lowest concentration of Ls (Diluted 100x from OD of 0.3) (8.75 mm or 175.00 AU/mL), the response of this combination was shown to be quite effective when this combination was evaluated against Ls in the highest concentration (OD 0.3) by the agar diffusion method (15.50 mm or 310.00 Au/mL) and when it was applied direct in the food (ready-to eat pork ham) (1.89 ± 0.26 log CFU/g) (Table 2).

These results are somewhat equivalent from that in the literature, once several bacteriocins show better synergism when used in combination with other antimicrobials, including nisin that had potentiated action in combinations with other antimicrobials such as chemical preservatives, phenolic compounds, other natural antimicrobial proteins and organic acid (Franklin, Cooksey, & Getty, 2004; Sant'Anna et al., 2013; Figueiredo & Almeida, 2017). The effectiveness of bacteriocins is often dependent upon environmental factors such as pH and temperature, interactions with food components, precipitation, preparation, inactivation, or uneven distribution of bacteriocin in the food matrix, and food microbiota (Grisi & Gorlach, 2005; Figueiredo & Almeida, 2017). These observations may support the findings in our study. The BLIS alone was not effective, but the combination with NISA showed sometimes some improvement (Table 2).

3.3. BLIS effects on weight loss and acidification

For weight loss (WL) over the shelf life time (d10), there was significant effect ($p < 0.05$) on WL of BLIS-treated samples (Table 2). The pork ham artificially contaminated with Lse and stored in vacuum package at 4ºC (control) presented WL of 3.00% ± 0.12, while in the treatments groups the lowest WL was obtained in BLIS-treated samples (2.77% ± 0.23) and the highest WL was presented in COMB-treated samples (3.20% ± 0.13). NISA-treated samples showed an intermediate WL regarding to the others (3.10% ± 0.21). The WL is influenced by the water retention capacity in meat structures (Silva Sobrinho et al., 2005). Therefore, according to these results, it is possible to conclude that BLIS-treated samples had a higher water retention capacity, which may justify this lower WL during storage. The pH is another important factor related to WL, in which lower values favor water loss, amount of intramuscular fat (barrier to water diffusion) and moisture content of the meat (Toldrá, 2002).

The pH values (Table 2) remained almost constant. In the control, NISA and COMB treatment, these values were not lower than 6.0, ranging from 6.0 to 6.2, meanwhile in the BLIS-treated samples they were the lowest among the groups, ranging from 5.9 to 5.8. The final pH value of the BLIS-treated samples was less than 6.0 however; the pH variation in this group (between 5.9 and 5.8) during the storage days (d0, d2, d6 and d10) was lower than that observed in the other groups (Table 2), which possibly justify the non-interference of the pH in WL during these 10 days of storage in the BLIS-treated samples.

3.4. BLIS effect on lipid peroxidation and color stability

According to Table 2, MDA levels were affected by either antimicrobial treatment after the shelf life of ten days (d10). The highest amount of MDA was observed in the control group (1.25 ± 0.01 mg MDA/Kg) and the difference was substantial ($p < 0.05$) among the treated groups, in which the lowest value of MDA was presented in COMB group (0.60 ± 0.00 mg MDA/Kg). BLIS (0.63 ± 0.00 mg MDA/Kg) and NISA (0.66 ± 0.00 mg MDA/Kg) (Table 2) also showed low values of MDA, being intermediary between control and COMB treatment, suggesting a protective effect of these antimicrobials for lipid peroxidation in the ready-to-eat pork ham, independently if they were used in combination or separately. Lipid oxidation represents one of the major

factors causing the progressive deterioration of meat products quality, limiting the shelf life during storage (Chakchouk-Mtibaa et al., 2015) and as showed in Table 2, it can be reduced by the presence of these antimicrobial agents. These antimicrobials agents were important to the development of the oxidative process, since these compounds may be promoting hydrophobic interactions between the fatty acids chains, making the lipids no longer exposed to the oxidation and, therefore, to delay the oxidative process (Berset & Cuvelier, 1996).

The superficial color values recorded for the ready-to-eat pork ham samples in the presence or absence of antimicrobials are shown in Table 3. At the end of the storage time (d10), the L* values, referring to lightness, were noted to range from 64.66 to 66.64, but are not different ($p = 0.785$). The color remained acceptable during the storage and did not show significant changes until the end of the experiment. Control, NISA, COMB and BLIS-treated samples values remained close (66.64 ± 1.31; 66.57 ± 1.32; 66.20 ± 2.39; 64.66 ± 2.02, respectively). The value in the BLIS-treated samples may be explained by the higher water retention capacity and the lower liquid loss in the environment, once the proteolysis starts after 24 h (Koohmaraie, 2002). This statement is consistent with the data of weight loss (WL) presented in table 2, since BLIS-treated samples, on the last day of analysis (d10), presented the lowest rate of WL (2.77%).

Meat products with darker coloration and lower intensity of red usually correspond to products with high final pH (Andrade et al., 2010), which corresponds to the values of L* and a* observed in the control and NISA (66.64 ± 1.31, 6.73 ± 0.42; 66.57 ± 1.32, 6.28 ± 0.73, respectively) (Table 3), as these showed the highest pH values (6.11) (Table 2). However, differences were not statistically significant. The analysis of a* (redness) values (Table 3) showed that at the beginning of the experiment (d0) the values were small, independently of the group (control: 4.99 ± 0.36; BLIS: 5.55 ± 0.93; NISA: 4.28 ± 0.40; COMB: 4.72 ± 0.87). In contrast, at the end of the storage (d10) at 4^{o}C, samples presented high values of redness and there were no significant changes throughout the storage ($p=0.514$). The reduction in red color intensity was higher in control and NISA-treated samples (6.73 ± 0.42 and 6.28 ± 0.73, respectively), which could be presumably be attributed to the interdependence between lipid oxidation and color oxidation (Lynch & Faustman, 2000). TBARS values for control (1.25 mg MDA/Kg) and NISA-treated samples (0.66 mg MDA/Kg) were the highest (Table 2). A decrease in a* values corresponds to decreases in the redness of meat due to myoglobin oxidation and metmyoglobin formation (Kennedy et al., 2005; Chakckouk-Mtibaa et al.,

2017). In terms of b* values, referring to yellowness, there were no significant differences between the control and treated samples (p = 0.179) (Table 3). The higher the values of L*, a* and b* means the meat paler, more red and yellow, respectively (Monte et al., 2005).

The discoloration of meat products determined the end of shelf life of these products (Greene, 1969; Andrade et al., 2010). Identifying the right moment of changes in the color of the meat during the storage, determine an important factor of interest for industries and the distribution sector (Andrade et al., 2010). In this way, MacDougal (1994) proposal for the evaluation of this parameter have been used (Lee et al., 2005; Luciano et al., 2009), which is constituted by the calculation of the coordinates L*, a* and b*, determining the saturation index (C*) and the hue angle (h*). These parameters allow to determine the color intensity, saturation or estimate the real browning of the meat and, normally the meat discoloration process is accompanied by an increase in the C* and h* values during the storage (Lee et al., 2005). Regarding to the saturation index (C*), there was an interaction between the storage time and treatments evaluated. In table 3, the C* values during the storage (d0, d2, d6, d10) showed significant increase (p=0.001), indicating the important process of discoloration that occurred during this time. The highest C* values were presented by samples in the groups BLIS and NISA (13.97 ± 0.11 and 14.36 ± 1.02) and values from samples in the control and COMB groups presented close values but lower (13.39 ± 0.28 and 13.13 ± 0.01, respectively) than the previous ones. The h* angle is also presented in table 3. The angle was similar among all treatments and coincident between BLIS and COMB-treated samples after 10 days storage. Considering these data, it is possible to conclude that the application of BLIS and Nisaplin (NISA) was favorable in maintaining a better and more intense coloration of the ready-to-eat pork ham indicating their potential of application in this type of food.

Table 3. Effect of antimicrobial treatments on color parameters (L*, a*, b*, C*, h*) of artificially contaminated ready-to-eat pork ham in vacuum-package storage at 4ºC during shelf life of 10 days

Parameters	Days	Control	BLIS	NISA	COMB
L* value	0	69.40 ± 0.68^{a}	69.24 ± 3.05^{a}	71.32 ± 0.85^{a}	70.27 ± 1.27^{a}
	2	64.67 ± 3.05^{a}	65.78 ± 3.84^{a}	67.25 ± 2.31^{a}	67.58 ± 2.07^{a}
	6	66.40 ± 1.31^{a}	66.14 ± 1.24^{a}	64.55 ± 3.08^{a}	64.78 ± 2.34^{a}
	10	66.64 ± 0.82^{a}	64.66 ± 2.02^{a}	66.57 ± 1.32^{a}	66.20 ± 2.39^{a}
a* value	0	4.99 ± 0.36^{a}	5.55 ± 0.93^{a}	4.28 ± 0.40^{a}	4.72 ± 0.87^{a}
	2	7.23 ± 1.87^{a}	7.68 ± 2.26^{a}	6.35 ± 0.97^{a}	6.15 ± 0.97^{a}
	6	7.34 ± 0.74^{a}	7.16 ± 0.62^{a}	7.49 ± 2.11^{a}	7.68 ± 0.62^{a}
	10	6.73 ± 0.42^{a}	7.69 ± 1.36^{a}	6.28 ± 0.73^{a}	7.23 ± 0.96^{a}
b* value	0	10.51 ± 0.27^{a}	10.29 ± 0.45^{a}	10.46 ± 0.36^{a}	10.72 ± 1.04^{a}
	2	11.92 ± 0.35^{a}	11.10 ± 0.46^{a}	11.78 ± 0.66^{a}	12.17 ± 0.60^{a}
	6	10.50 ± 0.46^{a}	11.24 ± 0.72^{a}	11.32 ± 0.37^{a}	12.21 ± 0.59^{a}
	10	11.57 ± 0.50^{a}	11.67 ± 0.59^{a}	12.91 ± 0.59^{a}	10.96 ± 0.31^{a}
C* index	0	11.63 ± 0.06^{b}	11.69 ± 0.34^{c}	11.30 ± 0.03^{a}	11.71 ± 0.12^{c}
	2	13.94 ± 1.13^{k}	13.50 ± 1.28^{h}	13.38 ± 0.43^{g}	13.63 ± 0.05^{j}
	6	12.81 ± 1.07^{d}	13.32 ± 1.27^{f}	13.57 ± 0.22^{i}	14.42 ± 0.26^{m}
	10	13.39 ± 0.28^{g}	13.97 ± 0.11^{k}	14.36 ± 1.02^{l}	13.13 ± 0.01^{e}
h* angle	0	$1.13 \pm 0.06^{g,h}$	$1.08 \pm 0.34^{e,f}$	1.18 ± 0.03^{i}	$1.16 \pm 0.12^{h,i}$
	2	$1.03 \pm 1.07^{c,d}$	$0.97 \pm 1.27^{a,b}$	$1.08 \pm 0.22^{e,f}$	$1.10 \pm 0.26^{f,g}$
	6	0.96 ± 0.20^{a}	$1.00 \pm 0.07^{a,b,c,d}$	$0.99 \pm 1.23^{a,b}$	$1.01 \pm 0.02^{b,c,d}$
	10	$1.04 \pm 0.06^{d,e}$	$0.99 \pm 0.54^{a,b,c}$	$1.12 \pm 0.10^{f,g,h}$	$0.99 \pm 0.46^{a,b,c}$

Legend: BLIS = bacteriocin-like inhibitory substance, NISA = Nisaplin®, COMB = combination of BLIS and NISA (50%/50%). Values are the mean of triplicates plus its standard deviation). Different letters mean statistically significant difference among the values of the same parameter, according to the test of Tukey ($p < 0.05$).

4. Conclusion

Currently, nisin is the only bacteriocin widely used as a commercial food preservative that has been categorized by the World Health Organization as a food biopreservative. Due to the growing demand for food biopreservatives, identifying new substances with this same antimicrobial capacity as nisin is warranted. Our data suggest the potential effectiveness of the bacteriocin-like inhibitory substance (BLIS), produced and secreted by *Pediococcus pentosaceus* ATCC 43200 after fermentation of glucose-based Man, Rogosa and Sharp (MRS) medium, regarding quality parameters and as biopreservative for control of *Listeria seeligeri* NCTC 11289 in ready-to-eat pork ham. During the course of our experiments, BLIS not only maintain low listeria multiplication but also promoted lower weight loss and low lipid peroxidation while maintaining more intense and better coloration of ham samples. The antimicrobial activity of BLIS may be further improved (and perhaps even potentiated) after semi and/or total purification of this antimicrobial molecule while maintaining excellent quality parameters for the consumer.

FINAL CONCLUSIONS AND PROSPECTS

The studies developed in the chapters previously presented in this thesis demonstrated that *P. pentosaceus* ATCC 43200 is a promising bacteriocin-like inhibitory substance (BLIS) producer strain, which can grow and produce BLIS in the recommended culture medium MRS with highest antimicrobial activity, especially when supplemented with an additional nitrogen source, such as peptone. BLIS was clearly *Pediococcus* growth associated, depending on its total biomass formation. *P. pentosaceus* ATCC 43200 demonstrated to be adaptable to different culture conditions that was submitted, but preferentially producing higher BLIS activity and a more stable molecule under restricted oxygen conditions (stationary, microaerophilic or anaerobic conditions). Besides, the culture medium agitation (200 rpm) was also quite important. Hence, addiction of peptone to MRS medium was able to speed up *P. pentosaceus* exponential growth phase onset, reducing the time in 4 h compared with MRS without peptone, and also improved its generation time. Moreover, BLIS showed biopreservative efficiency in ready-to-eat ham pork artificially contaminated with *Listeria seeligeri* NCTC11289, indicating its potential to be used as a biopreservative in the food industry.

As a prospective, BLIS molecular weight and amino acid sequenced should be determine, for instance by mass spectrometry and by Fourier-transform infrared spectroscopy, and BLIS also should be purified in an attempt to further increase its efficiency as an antimicrobial. However, our research group will perform these steps in a future work.

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Appendix A – Published article: PharmaNutrition (IF: 0.71), 2018.

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Full Length Article

Production of fermented skim milk supplemented with different grape pomace extracts: Effect on viability and acidification performance of probiotic cultures

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ABSTRACT

The addition of polyphenolic compounds to probiotic dairy products has been proposed as a promising strategy to enhance the beneficial health effects of milk-derived functional foods. In this study, probiotic fermented skim milk was supplemented with pomace extracts obtained from Pinot Noir, Freisa, Croatina and Barbera grape varieties. Regarding acidification kinetics, the addition of Pinot Noir extract increased the maximum acidification rate (V_{max}) of skim milk by 39.4% compared with control (no pomace extract supplementation). The time required to complete the fermentation ($t_{pH4.5}$ = 3.5 h) was shortened when grape pomace extracts were added to the fermented skim milk. It was also observed that after 28 days of storage at 4 °C, polyphenolic compound supplementations had a positive effect on cell viability of both *Streptococcus thermophilus* and *Lactobacillus acidophilus*. The concentration of polyphenols was also determined in the fermented skim milk samples. These results suggest that *Streptococcus thermophilus* and *Lactobacillus acidophilus* can metabolize the supplemented polyphenols, although not all to the same extent. Moreover, this study demonstrates the feasibility of adding phenolic compounds to probiotic products in order to further improve their functional health properties.

1. Introduction

Lactic acid bacteria (LAB) have traditionally been associated with the fermentation of food and animal feed. LAB are one of the most important microorganisms used in food fermentation, with many LAB strains considered as probiotics. As living microorganisms, probiotics may provide health benefits to the host (when ingested in sufficient amounts) by improving the composition of intestinal microflora [1,2] and by crowding out pathogens that may otherwise cause disease [3].

In the *Streptococcus* genus, there are species recognized as pathogenic and others as probiotics. Whereas pathogenic *Streptococcus* species are associated with human and animal diseases, probiotic ones are important in the dairy industry [4,5]. *Streptococcus thermophilus*, for example, is one of the probiotic bacteria that play an important role in the texture of yogurts and other fermented dairy products [6], especially by the production of exopolysaccharides (EPS) [7]. According to Zhang et al. [8], *S. thermophilus* is responsible for the stabilization effect of EPS on the textural and microstructural properties of fermented skim milk.

Probiotic microorganisms are commonly added into dairy products to provide functional health effects [9]. For instance, the addition of the probiotic *Lactobacillus acidophilus* 593 N to cheese may provide health benefits to consumers through their antagonistic effect against food-borne disease agents, including *Enterococcus faecium* and *Listeria monocytogenes* [10]. In dairy products, the use of co-cultures is very common (e.g., probiotic *Streptococcus* combined with different *Lactobacillus* strains) causing a symbiotic effect. In fact, several authors have observed a more pronounced positive activity of co-cultures in comparison with monocultures in terms of growth, acidification, production of flavor, EPS and proteolysis [11,12].

Grape (*Vitis vinifera*) is one of the world's most important fruit crops, with a global production of around 73 million tons in 2015, of which 274.7 mhl were used to produce wine [13]. This industry generates an enormous amount of biomass, known as pomace, which include grape

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Research review paper

Pediococcus spp.: An important genus of lactic acid bacteria and pediocin producers

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ABSTRACT

Probiotics have gained increasing attention due to several health benefits related to the human digestive and immune systems. *Pediococcus* spp. are lactic acid bacteria (LAB) that are widely described as probiotics and characterized as coccus-shaped bacteria (arranged in tetrads), Gram-positive, non-motile, non-spore forming, catalase-negative, and facultative anaerobes. There are many *Pediococcus* strains that produce pediocin, an effective antilisterial bacteriocin. Pediocins are small, cationic molecules consisting of a conserved hydrophilic N-terminal portion containing the YGNGV motif and an amphiphilic or hydrophobic C-terminal variable portion. A number of studies have been developed with *Pediococcus* isolated from multiple biological niches to conduct fermentation processes for pediocin or *Pediococcus* cell production. This review gathers the most significant information about the cultivation, mode of action, and variability of bacteriocins produced by *Pediococcus* spp., emphasizing their applications in the areas of food and clinical practice. This updated panorama assists in delimiting the challenges that still need to be overcome for pediocin use to be approved for human consumption and the food industry.

Contents

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Influence of temperature and pH on the production of biosurfactant, bacteriocin and lactic acid by *Lactococcus lactis* CECT-4434

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ABSTRACT

Operational conditions such as temperature and pH are well known to influence the production of biosurfactants, bacteriocins, and lactic acid by lactic acid bacteria. The bacterial strain *Lactococcus lactis* subsp. *lactis* CECT-4434 was used to investigate the effects of temperature (30°C, 37°C and 40°C) and pH control on the production of these biomolecules, in order to establish whether they are able to preferentially address its metabolism towards one product or another. It was observed that the pH control within the range 5.0–5.3 favored the production of lactic acid, which was 38% higher than that obtained without control. The bacteriocin production was higher in the temperature range between 30°C and 37°C, evidenced by the formation of inhibition zones against *Lactobacillus sakei* and *Staphylococcus aureus*, 5–22% and 4–14% higher, respectively, compared with those achieved at 40°C. Finally, the best results for biosurfactant synthesis occurred at 37°C without pH control, with a surface tension reduction between 22.5 and 24.7 mN/m after 6–24 h of culture, respectively.

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Influencia de la temperatura y pH en la producción de biosurfactantes, bacteriocinas y ácido láctico por *Lactococcus lactis* CECT-4434

RESUMEN

Es bien conocido que determinadas condiciones operacionales, tales como la temperatura y el pH, influyen en la producción de biosurfactantes, bacteriocinas y ácido láctico por parte de las bacterias ácido lácticas. En este trabajo se empleó la cepa bacteriana *Lactococcus lactis* subsp. *lactis* CECT-4434 para investigar los efectos de la temperatura (30, 37 y 40°C) y el control del pH en la producción de estas biomoléculas, con el fin de determinar como influyen en su metabolismo. Se observó que a pH controlado, dentro del rango 5,0–5,3, se favorecía la producción de ácido láctico, que resultó 38% superior a la obtenida sin control. La producción de bacteriocina fue mayor dentro del rango de temperatura 30–37°C, evidenciada por la formación de zonas de inhibición frente a las cepas indicadoras *Lactobacillus sakei* y *Staphylococcus aureus*, 5–22% y 4–14% respectivamente superiores que aquellos valores alcanzados a 40°C. Finalmente, se observaron los mejores resultados para la síntesis de biosurfactantes a 37°C sin control de pH, con una reducción de la tensión superficial entre 22,5 y 24,7 mN/m tras 6–24 h de cultivo respectivamente.

1. Introduction

Lactic acid bacteria (LAB) are Gram-positive cocci or rods, which are Generally Recognized as Safe by the FDA (US Food and Drug Administration) and produce lactic acid as the major or sole fermentation product (Von Wright & Axelsson, 2012). They comprise a large number of species involved in the production of fermented milk products that belong mainly to the genera *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus,* and *Streptococcus* (Mayo et al., 2010).

It is important to note that although lactic acid is the major metabolite produced by LAB, other biomolecules can simultaneously be produced depending on the species such as peptides called bacteriocins (Arauz, Jozala, Mazzola, & Penna, 2009). Bacteriocins produced by LAB are proteins or protein complexes with antimicrobial activity and therapeutic potential, even against the cancer (Moreno, Lerayer, Baldini, & Leitão, 2000; Panesar, Kennedy, Gandhi, & Bunko, 2007; Parada, Caron, Medeiros, & Soccol, 2007). The economic importance of bacteriocins in food preservation has increased their exploitation as food preservatives (Rosa & Franco, 2002).

Currently, the most widely studied and industrially exploited bacteriocin is nisin, which is produced by certain strains of the probiotic bacterium *Lactococcus lactis*, is classified as a lantibiotic, contains 34 amino acids and has a molecular weight of 3.5 kDa (Arauz et al., 2009; Oladunjoye, Singh, & Ijabadeniyi, 2016). Nisin is able to inhibit pathogens and contaminants and is the only bacteriocin internationally legalized for use as a natural biopreservative of foods, since its intake does not exert toxic effects to the human body, does not alter the ecology of the gastrointestinal tract, and, then, does not present the same risks related to the use of antibiotics (Yoneyama & Katsumata, 2006).

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Appendix D – Published article in collaboration: Biochemical Engineering Journal (IF: 2.89).

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Regular article

Optimization of biosurfactant and bacteriocin-like inhibitory substance (BLIS) production by *Lactococcus lactis* CECT-4434 from agroindustrial waste

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Central composite design

ABSTRACT

The use of low-cost substrates and wastes as fermentative media is a simple strategy for overcoming excessive production costs of bioactive molecules and to make the process more profitable. In this context, the aim of this work was to optimize the production of biosurfactant by *Lactococcus lactis* CECT-4434 from whey and vinasse from industrial sources. For this purpose, a 2^4 central composite design (CCD) was proposed to maximize biosurfactant production. Besides, the ability of *Lc. lactis* to synthesize bacteriocin-like inhibitory substance (BLIS) was also evaluated, therefore contributing to the profitability of the biotechnological process. Results showed that whey at 15% positively influenced biosurfactant production (on average 8.9 mg/L), reducing surface tension by about 18.1 mN/m. Molecular composition of the biosurfactant was determined by Fourier transform infrared spectroscopy and nuclear magnetic resonance which confirmed the presence of carbohydrates, lipids and proteins, defining this molecule as a glycolipopeptide. Regarding the BLIS synthesis, the highest concentration of sucrose (2.0%) and the lowest vinasse level (1.0%) reduced the production of BLIS identified by the decrease in antimicrobial activity (mean inhibition zone of 10.7 mm). Conversely, the lowest sucrose concentration and highest vinasse level exerted the strongest inhibition, with a mean inhibition zone of 12.2 mm against *Staphylococcus aureus* CECT-239.

1. Introduction

The search for novel and natural surfactants that outperform the chemical surfactants activity, while presenting biodegradability and low to no toxicity, has been driving the research efforts in the field [1,2]. In addition, biosurfactants are more compatible with the environment, are stable under extreme temperature conditions, pH and salinity [3,4], presenting low critical micellar concentrations and better surface activity than their chemical counterparts [5].

Biosurfactants produced by microorganisms (bacteria, fungi, and yeast) are found to have a wide range of applications in environmental protection, which include enhancing oil recovery, controlling oil spills, biodegradation, and detoxification of oil-contaminated industrial effluents and soils [6–8]. Also, these surface-active biomolecules have potential applications in pharmaceutical/medicine, food, cosmetic, pesticide, oil, and biodegradation industries [8].

In spite of the numerous attractive properties and advantages of biosurfactants at an industrial, the production of biosurfactants in the industrial scale has not been a reality due to the high investment costs [9–11], not being able to economically compete with synthetic surfactants [11]. The commercialization is every time more limited to some biosurfactants, such as surfactin [12], sophorolipids, rhaminolipids and mannosylerythritol lipids [13].

To overcome this limitation and enlarge the commercial use of biosurfactants, research efforts have been focused on the reduction of the production costs [14]. Saharan et al. [15] described the use of low-cost substrates and wastes as fermentative media as a strategy for overcoming excessive biosurfactants production costs. The choice of low-cost raw materials has a great impact on the process economics, as these represent around 50% of the final cost of the product [11,16].

The industry of dairy products produces a considerable amount of subproducts, such as butter milk, whey, and its derivatives. The use of whey as substrate to culture fermentative organ-

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ATTACHMENT A – Statement for exemption of Ethics Committee approval.

UNIVERSIDADE DE SÃO PAULO

Faculdade de Ciências Farmacêuticas

Departamento de Tecnologia Bioquímico-Farmacêutica

DECLARAÇÃO DE DISPENSA DE AUTORIZAÇÃO DE COMITÊ DE ÉTICA

São Paulo, 16 de Fevereiro de 2018

Eu, Pamela Oliveira de Souza de Azevedo Nº USP 6384841, aluna de doutorado 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 **Otimização do rendimento de substância semelhante a bacteriocina (BLIS) produzido por *Pediococcus pentosaceus* e sua aplicação como bioconservante de alimentos**, dispensa a autorização pelo Comitê de Ética visto que não serão envolvidos animais ou seres humanos para a realização dos experimentos.

Pamela Oliveira de Souza de Azevedo

Pamela Oliveira de Souza de Azevedo

Ricardo P. S. Oliveira

Prof. Dr. Ricardo Pinheiro de Azevedo

ATTACHMENT B – Student file

Janus - Sistema Administrativo da Pós-Graduação

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Documento sem validade oficial
FICHA DO ALUNO

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Curso:	Doutorado
Programa:	Tecnologia Bioquímico-Farmacêutica
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Início da Contagem de Prazo:	30/04/2014
Data Limite para o Depósito:	02/05/2018
Orientador:	Prof(a). Dr(a). Ricardo Pinheiro de Souza Oliveira - 30/04/2014 até o presente. Email: rpsolive@usp.br
Proficiência em Línguas:	Inglês, Aprovado em 30/04/2014
Data de Aprovação no Exame de Qualificação:	Aprovado em 01/07/2016
Estágio no Exterior:	Universität für Bodenkultur Wien, Áustria - Período de 03/04/2017 até 02/10/2017
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 30/04/2014

Aluno matriculado no Regimento da Pós-Graduação USP (Resolução nº 5473 em vigor de 18/09/2008 até 19/04/2013).
Última ocorrência: Matrícula de Acompanhamento em 06/02/2017
Impresso em: 15/02/2018 07:54:10

Janus - Sistema Administrativo da Pós-Graduação

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

9134 - 6384841/1 - Pamela Oliveira de Souza de Azevedo

Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação
MIP5737-2/2	Fundamentos da Citometria de Fluxo - Aplicações na Investigação da Relação Hospedeiro-Parasita (Faculdade de Medicina - Universidade de São Paulo)	02/06/2014	08/06/2014	30	2	100	C	N	Concluída
FBT5737-7/1	Tecnologia de Síntese de Fármacos	21/08/2014	22/10/2014	90	0	-	-	N	Turma cancelada
ICB5736-1/1	Bioinformática Prática (Instituto de Ciências Biomédicas - Universidade de São Paulo)	15/09/2014	24/11/2014	60	0	-	-	N	Pré-matrícula indeferida
MCM5851-4/2	Adesão Celular e Câncer (Faculdade de Medicina - Universidade de São Paulo)	08/10/2014	09/12/2014	90	6	80	B	N	Concluída
FBT5781-4/2	Culturas Probióticas: Aplicações Tecnológicas	21/10/2014	13/11/2014	60	0	-	-	N	Matrícula cancelada
MIP5742-1/2	Citometria de Fluxo Avançada - Aplicações na Investigação da Relação Hospedeiro-Parasita (Faculdade de Medicina - Universidade de São Paulo)	30/10/2014	10/12/2014	90	6	90	B	N	Concluída
FBT5773-7/5	Tópicos Especiais em Tecnologia Bioquímico-Farmacêutica	02/03/2015	10/05/2015	30	0	-	-	N	Pré-matrícula indeferida
FBT5713-2/1	Biologia Molecular Aplicada à Biotecnologia Farmacêutica Industrial	09/03/2015	22/03/2015	60	4	100	A	N	Concluída
FBA5897-2/2	Nutrigenômica do Câncer	03/08/2015	09/08/2015	30	2	100	A	N	Concluída
FBT5773-7/6	Tópicos Especiais em Tecnologia Bioquímico-Farmacêutica	03/08/2015	19/10/2015	30	2	100	A	N	Concluída
FBC5719-3/2	Trato Gastrointestinal: Imunomodulação da Colonização e Infecção Bacteriana	10/08/2015	22/11/2015	90	6	100	A	N	Concluída
BTC5704-7/3	Engenharia Bioquímica (Curso Interunidades: Biotecnologia - Universidade de São Paulo)	29/09/2015	11/01/2016	75	0	-	-	N	Matrícula cancelada
ICB5709-6/3	Ensaios Pedagógicos (Instituto de Ciências Biomédicas - Universidade de São Paulo)	04/11/2015	08/12/2015	45	3	100	A	N	Concluída
FBT5787-1/4	Aplicação Biotecnológica de Bactérias Láticas	01/02/2016	06/03/2016	45	3	100	A	N	Concluída
FBT5768-5/1	Princípios de Fermentação Contínua	26/04/2016	27/06/2016	75	5	100	A	N	Concluída

	Créditos mínimos exigidos		Créditos obtidos
	Para exame de qualificação	Para depósito de tese	
Disciplinas:	0	20	39
Estágios:			
Total:	0	20	39

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: 15/02/2018 07:54:10

ATTACHMENT C – Curriculum lattes

Pamela Oliveira de Souza de Azevedo

Endereço para acessar este CV: http://lattes.cnpq.br/9916074595995312

Última atualização do currículo em 18/02/2018

possui graduação em Biomedicina pela Universidade Bandeirante de São Paulo - UNIBAN (2006) e mestrado em Ciências Médicas pela Faculdade de Medicina da USP (2011). Atualmente é doutoranda na área de Tecnologia de Fermentações no departamento de Tecnologia Bioquímico-Farmacêutica da Faculdade de Ciências Farmacêuticas da USP. Tem experiência na área de oncologia, com ênfase em oncohematologia e farmacogenética. Atua, sobretudo, nos seguintes temas: bactérias láticas, probióticos, prebióticos, produção de biomoléculas microbianas por processos fermentativos e efeito de biomoléculas microbianas com ênfase na área de alimentos e farmacêutica. **(Texto informado pelo autor)**

Identificação

Nome Pamela Oliveira de Souza de Azevedo

Nome em citações bibliográficas Oliveira-Souza, P.;Souza, P.O.;Azevedo, P.O.S.;Souza-Azevedo, P.O.;Azevedo, Pamela Oliveira de Souza de;De Souza de Azevedo, Pamela Oliveira;De Azevedo, Pamela Oliveira de Souza

Endereço

Formação acadêmica/titulação

2014 Doutorado em andamento em Tecnologia de Fermentações.
Faculdade de Ciências Farmacêuticas da USP, FCF-USP, Brasil.
com **período sanduíche** em Universität für Bodenkultur Wien, Viena, Áustria (Orientador: Univ. Prof. Dr. Martin Gierus).
Título: Otimização do rendimento de substância semelhante a bacteriocina (BLIS) produzida por Pediococcus pentosaceus e sua aplicação como bioconservante de alimentos,
Orientador: Prof. Dr. Ricardo Pinheiro de Souza Oliveira.
Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES, Brasil.
Palavras-chave: Pediococcus pentosaceus; produção biotecnológica; bactérias ácido láticas; fermentação (prebióticos e probióticos); bacteriocina (pediocina); células do sistema imune.
Grande área: Ciências Biológicas
Grande Área: Ciências Biológicas / Área: Microbiologia / Subárea: Microbiologia Industrial e de Fermentação.
Grande Área: Ciências da Saúde / Área: Farmácia / Subárea: Farmácia (biofármacos).

2007 - 2010 Mestrado em Ciências Médicas.
Universidade de São Paulo, Faculdade de Medicina, FMUSP, Brasil.
Título: Polimorfismos de enzimas de fase 1 e 2 do metabolismo de drogas de pacientes portadores de linfoma difuso de grandes células B.,Ano de Obtenção: 2011.
Orientador: Dr. Sérgio Paulo Bydlowski.
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq, Brasil.
Palavras-chave: Polimorfismos de enzimas do metabolismo; Linfoma Difuso de Grandes Células B; CHOP; R-CHOP.

2003 - 2006 Graduação em Biomedicina.
Universidade Bandeirante de São Paulo, UNIBAN, Brasil.

Atuação Profissional

Universidade de São Paulo, Faculdade de Medicina, FMUSP, Brasil.

Vínculo institucional

2007 - 2010 Vínculo: Pós-graduação, Enquadramento Funcional: Pós-graduanda, Regime: Dedicação exclusiva.

Universidade Federal de São Paulo, UNIFESP, Brasil.

Vínculo institucional

2006 - 2007 Vínculo: Livre, Enquadramento Funcional: Estagiária, Regime: Dedicação exclusiva.

Atividades

04/2006 - 05/2007 Estágios , Departamento de Morfologia, Disciplina de Genética.

Estágio realizado
Citogenética e Genética Molecular.

12/2003 - 11/2004 Estágios , Departamento de Microbiologia Imunobiologia e Parasitologia, Disciplina de Imunologia.

Estágio realizado
Biologia Celular e Análise Toxicológica de Compostos Sintéticos em Melanoma.

Instituto Butantan / SP, BUTANTAN, Brasil.

Vínculo institucional

2004 - 2006 Vínculo: Livre, Enquadramento Funcional: Estagiária, Regime: Dedicação exclusiva.

Atividades

11/2004 - 02/2006 Estágios , Laboratório de Bioquímica e Biofísica, .

Estágio realizado
Biologia Celular e Análise Farmacológica - Toxicológica de Compostos Sintéticos em Linhagens Tumorais.

Universidade de São Paulo, Faculdade de Ciências Farmacêuticas, USP, FCF, Brasil.

Vínculo institucional

2014 - Atual Vínculo: Bolsista, Enquadramento Funcional: Doutoranda, Regime: Dedicação exclusiva.

Outras informações Projeto de pesquisa: produção otimizada de bacteriocina produzida por cepa de bactéria ácido-lática considerada probiótica e sua aplicação na área de alimentos e farmacêutica.

Revisor de periódico

2014 - Atual Periódico: Journal of Bacteriology and Mycology

2014 - Atual Periódico: Food Microbiology (Print)

2015 - Atual Periódico: International Journal of Food Science and Technology

2015 - Atual Periódico: Clinics (USP. Impresso)

Áreas de atuação

1. Grande área: Ciências Biológicas / Área: Biologia Geral / Subárea: Biologia celular e molecular.
2. Grande área: Ciências da Saúde / Área: Farmácia / Subárea: Oncologia.
3. Grande área: Ciências da Saúde / Área: Engenharia Biomédica / Subárea: Biotecnologia.
4. Grande área: Ciências Biológicas / Área: Microbiologia / Subárea: Bacteriocina.
5. Grande área: Ciências Biológicas / Área: Microbiologia / Subárea: Bactéria ácido lática.
6. Grande área: Ciências Biológicas / Área: Microbiologia / Subárea: Fermentação (prebiótico e probiótico).

Idiomas

Inglês Compreende Bem, Fala Bem, Lê Bem, Escreve Razoavelmente.

Espanhol Compreende Pouco, Fala Pouco, Lê Pouco, Escreve Pouco.

Alemão Compreende Razoavelmente, Fala Razoavelmente, Lê Razoavelmente, Escreve Razoavelmente.

Prêmios e títulos

2011 Mestre em Ciências Médicas, Faculdade de Medicina da USP.

2005 Prêmio de Incentivo ao Jovem Pesquisador - Iniciação Científica - da VII Reunião Científica Anual do Instituto Butantan (primeira colocada), CNPq - PIBIQ.

Produções

Produção bibliográfica

Artigos completos publicados em periódicos

Ordenar por

Ordem Cronológica

1. **Azevedo, P.O.S.**; MOLINARI, F. ; OLIVEIRA, R.P.S. . Importance of the agar-media in the evaluation of bacteriocin activity against the same test-microorganisms.. BRAZILIAN JOURNAL OF PHARMACEUTICAL SCIENCES (ONLINE) JCR, v. 54, p. xx-xx, 2018.

2. doi VERA, ELLEN CRISTINA SOUZA ; **De Azevedo, Pamela Oliveira de Souza** ; DOMÍNGUEZ, JOSÉ MANUEL ; OLIVEIRA, RICARDO PINHEIRO DE SOUZA . Optimization of biosurfactant and bacteriocin-like inhibitory substance (BLIS) production by Lactococcus lactis CECT-4434 from agroindustrial waste. BIOCHEMICAL ENGINEERING JOURNAL JCR, v. x, p. xx, 2018.

3. doi **De Souza de Azevedo, Pamela Oliveira**; ALIAKBARIAN, BAHAR ; CASAZZA, ALESSANDRO ALBERTO ; LEBLANC, JEAN GUY ; PEREGO, PATRIZIA ; DE SOUZA OLIVEIRA, RICARDO PINHEIRO . Production of fermented skim milk supplemented with different grape pomace extracts: effect on viability and acidification performance of probiotic cultures. PHARMANUTRITION, v. xx, p. 1-22, 2018.

4. doi SOUZA, ELLEN CRISTINA ; **Azevedo, Pamela Oliveira de Souza de** ; DOMÍNGUEZ, JOSÉ MANUEL ; CONVERTI, ATTILIO ; OLIVEIRA, RICARDO PINHEIRO DE SOUZA . Influence of temperature and pH on the production of biosurfactant, bacteriocin and lactic acid by *Lactococcus lactis* CECT-4434. CyTA-Journal of Food JCR, v. 15, p. 525-530, 2017.

5. doi **De Souza de Azevedo, Pamela Oliveira**; CONVERTI, ATTILIO ; DOMÍNGUEZ, JOSÉ MANUEL ; DE SOUZA OLIVEIRA, RICARDO PINHEIRO . Stimulating Effects of Sucrose and Inulin on Growth, Lactate, and Bacteriocin Productions by Pediococcus pentosaceus. Probiotics and Antimicrobial Proteins JCR, v. 9, p. 466-472, 2017.

6. doi PORTO, MARIA CAROLINA W. ; KUNIYOSHI, TAÍS MAYUMI ; **Azevedo, P.O.S.** ; VITOLO, MICHELE ; OLIVEIRA, R.P.S. . Pediococcus spp.: An important genus of lactic acid bacteria and pediocin producers. BIOTECHNOLOGY ADVANCES JCR, v. 35, p. 361-374, 2017.

7. ★ LEVY, D. ; BELESSO, M. ; **Oliveira-Souza, P.** ; MACIEL, F.V.R. ; PEREIRA, J. ; BYDLOWSKI, S. P. . Fcgamma RIIA polymorphism and survival of patients with diffuse large B-cell lymphoma (DLBCL) treated with rituximab and CHOP. Study in a genetic admixed population. Clinics (USP. Impresso) JCR, v. 66, p. 919-922, 2011.
   Citações: WEB OF SCIENCE™ 2 | SciELO 4 | SCOPUS 6

8. Vassão. R.C. ; **Oliveira-Souza, P.** ; Festaneto, C. ; Maria, D.A. . Effects of imiquimod treatment on experimental melanoma. ARBS. Annual Review of Biomedical Sciences (Online), v. 7, p. 07-08, 2005.

Capítulos de livros publicados

1. ★ BYDLOWSKI, S. P. ; **Oliveira-Souza, P.** ; MASELLI, L.M.F. . Bases da Farmacogenética, em fase de impressão.. In: Antonio Carlos Lopes. (Org.). Diagnóstico e Tratamento em Clínica Médica.. 34ed.São Paulo: Atheneu, 2013, v. , p. 5892-5905.

2. ★ BYDLOWSKI, S. P. ; DEBES, A. A ; **Souza, P.O.** . Farmacogenética nas doenças hematológicas.. In: Milton de Arruda Martins; Flair José de Carrilho; Venâncio Avancini Ferreira Alves; Euclides Ayres de Castilho; Giovanni Guido Cerri; Chao Lung Wen.. (Org.). Clínica Médica.. São Paulo: Manole, 2009, v. 3, p. 44-50.

3. ★ BYDLOWSKI, S. P. ; DEBES, A. A ; **Souza, P.O.** . Farmacogenética.. In: Antonio Carlos Lopes. (Org.). Tratado de Clínica Médica.. São Paulo: Editora Roca Ltda, 2009, v. 3, p. 5278-5282.

Resumos publicados em anais de congressos

1. **Azevedo, P.O.S.**; OLIVEIRA, R.P.S. . Discontinuous fermentation process by probiotic bacterium to produce antimicrobial peptide with potential application as food preservative. In: 16. BOKU Symposium Tierenährung, 2017, Viena, Áustria. 16. BOKU Symposium Tierenährung, 2017.

2. **Azevedo, P.O.S.**; PIAZENTIN, A. C. M. ; OLIVEIRA, R.P.S. . Discontinuous fermentation process by probiotic bacterium to produce antimicrobial peptide witn potential application as food preservative. In: 29 Congresso Brasileiro de Microbiologia, 2017, Foz do Iguaçu. 29 Congresso Brasileiro de Microbiologia, 2017.

3. **Azevedo, P.O.S.**; ALIAKBARIAN, B. ; CASAZZA, A. A. ; LeBLANC, J. G. ; PEREGO, P. ; OLIVEIRA, R.P.S. . Effect of grape pomace extracts on Lactobacillus acidophylus and Streptococcus thermophylus viability and acidification kinetics. In: LABsymposium, 2017, Holanda. LABsymposium, 2017.

4. **Azevedo, P.O.S.**; OLIVEIRA, R.P.S. . Effect of Carbohydrates Sources on Growth, Lactate and Bacteriocin Productions by Pediococcus pentosaceus. In: XXI Semana Farmacêutica de Ciência e Tecnologia, 2016, São Paulo. XXI Semana Farmacêutica de Ciência e Tecnologia, 2016.

5. Farinha, L. R. L. ; PIAZENTIN, A. C. M. ; **Azevedo, P.O.S.** ; SOUZA, ELLEN CRISTINA ; OLIVEIRA, R.P.S. . ACIDIFICATION KINETIC AND GROWTH OF STREPTOCOCCUS THERMOPHILUS TA 040 AND LACTOCOCCUS LACTIS CECT 4434 FROM WHEY. In: 28 Congresso Brasileiro de Microbiologia, 2015, Florianópolis. 28 Congresso Brasileiro de Microbiologia, 2015.

6. **Azevedo, P.O.S.**; OLIVEIRA, R.P.S. . Effect of Inulin and Sucrose on Growth and Bacteriocin Productiion by Pediococcus pentosaceus. In: XX Semana Farmacêutica de Ciência e Tecnologia, 2015, São Paulo. XX Semana Farmacêutica de Ciência e Tecnologia, 2015.

7. SABO, S. S. ; **Azevedo, P.O.S.** ; OLIVEIRA, R.P.S. . Effect of Different Prebiotics on Growth and Bacteriocin Production by Lactobacillus plantarum. In: XX Semana Farmacêutica de Ciência e Tecnologia, 2015, São Paulo. XX Semana Farmacêutica de Ciência e Tecnologia, 2015.

8. PORTO, MARIA CAROLINA W. ; **Azevedo, P.O.S.** ; VITOLO, MICHELE ; OLIVEIRA, R.P.S. . Effect of pH and Polydextrose supplementation in Pediococcus pentosaceus ATCC 43200 growth and bioproducts synthesis during shaked-growing. In: XX Semana Farmacêutica de Ciência e Tecnologia, 2015, São Paulo. XX Semana Farmacêutica de Ciência e Tecnologia, 2015.

9. **Azevedo, P.O.S.**; Farinha, L. R. L. ; OLIVEIRA, R.P.S. . EFFECT OF INULIN AND SUCROSE ON GROWTH AND BACTERIOCIN PRODUCTION BY PEDIOCOCCUS PENTOSACEUS. In: 28 Congresso Brasileiro de Microbiologia, 2015, Florianópolis. 28 Congresso Brasileiro de Microbiologia, 2015.

10. **Souza, P.O.**; LEVY, D. ; MACIEL, F.V.R. ; XAVIER, F.D. ; PEREIRA, J. ; BYDLOWSKI, S. P. . NQO1 gene polymorphisms in patients with diffuse large B cell lymphoma. Relationship with outcome and response to treatment.. In: XXXIII World Congress of the International Society of Hematolog, 2010, Jerusalém. Annals, 2010, 2010.

11. **Souza, P.O.**; LEVY, D. ; BOOIJ, H. G. ; MACIEL, F.V.R. ; GRAMPAOLI, V. ; PEREIRA, J. ; BYDLOWSKI, S. P. . Genetic polimorphisms of glutathione-S-transferase genes. Association with overall response/clinical features in diffuse large B-cell lymphoma patients treated with rituximab.. In: 4o Simpósio "Avanços em Pesquisa Médica dos Laboratórios de Investigação Médica do HCFMUSP, 2010, São Paulo. Clinics, 2010., 2010. v. 65. p. S72.

12. LEVY, D. ; **Souza, P.O.** ; BELESSO, M. ; MACIEL, F.V.R. ; PEREIRA, J. ; BYDLOWSKI, S. P. . Survival of patients with diffuse large B-cell lymphoma treated with rituximab. Lack of association with FcRIIA polymorphism.. In: 4o Simpósio "Avanços em Pesquisa Médica dos Laboratórios de Investigação Médica do HCFMUSP, 2010, São Paulo. Clinics, 2010., 2010. v. 65. p. S65.

13. BYDLOWSKI, S. P. ; **Souza, P.O.** ; LEVY, D. ; BOOIJ, H. G. ; MACIEL, F.V.R. ; PEREIRA, J. . Association of polymorphisms of glutatione-S-transferase (GST) genes with overall treatment response/clinical features in diffuse large B-cell lymphoma patients.. In: 39th Annual Scientific Meeting of the Society for Hematology and Stem Cells, 2010, Melbourne, Australia.. Experimental Hematology, 2010., 2010. v. 38. p. S67.

14. LEVY, D. ; **Souza, P.O.** ; MACIEL, F.V.R. ; XAVIER, F.D. ; PEREIRA, J. ; BYDLOWSKI, S. P. . Polimorfismos de enzimas de fase 1 e 2 do metabolismo de fármacos em pacientes portadores de linfoma difuso de grandes células B.. In: 56o. Congresso Brasileiro de Genética, 2010, Guarujá, SP. Anais, 2010., 2010.

15. LEVY, D. ; **Oliveira-Souza, P.** ; BELESSO, M. ; MACIEL, F.V.R. ; PEREIRA, J. ; BYDLOWSKI, S. P. . FCγRIIa polymorphism is not associated with overall response in diffuse lwrge B cell Lymphoma treated with rituximab.. In: 51st ASH annual meeting and exposition, 2009, New Orleans, LA. 2009 Annual Meeting Abstracts, 2009., 2009.

16. **Oliveira-Souza, P.**; LEVY, D. ; BOOIJ, H. G. ; MACIEL, F.V.R. ; PEREIRA, J. ; BYDLOWSKI, S. P. . Association of genetic polymorphisms of Glutatione-S-transferase Genes and overall response/clinical features in diffuse large-B-cell lymphoma patients treated with rituximab.. In: ASH Annual Meeting and exposition, 2009, New Orleans, LA. 2009 Annual Meeting Abstracts on CD-ROM, 2009., 2009.

17. **Oliveira-Souza, P.**; LEVY, D. ; MACIEL, F.V.R. ; PEREIRA, J. ; BYDLOWSKI, S. P. . Características polifórmicas do gene MDR1 em pacientes com linfoma difuso de grandes células B.. In: Hemo 2009- Congresso Brasileiro de Hematologia e Hemoterapia., 2009, Florianópolis -SC. Revista Brasileira de Hematologia e Hemoterapia. Rio de Janeiro: Trasso, 2009. v. 31. p. 149-149.

18. **Oliveira-Souza, P.**; BOOIJ, H. G. ; LEVY, D. ; MACIEL, F.V.R. ; PEREIRA, J. ; BYDLOWSKI, S. P. . Poliformismo do gene da glutationa-S-Transferase (GSTM1, GSTT1 e GSTP1) em pacientes com linfoma de grandes células B: Implicação teapêutica e de prognóstico.. In: Hemo 2009 Congresso Brasileiro de Hematologia e Hemoterapia., 2009, Florianópolis, SC. Revista Brasileira de Hematologia e Hemoterapia.. Rio de Janeiro: Trasso, 2009. v. 31. p. 161-162.

19. **Souza, P.O.**; LEVY, D. ; MASELLI, L.M.F. ; BYDLOWSKI, S. P. . Polimorfismo no gene MDR1 em pacientes com linfoma difuso de grandes células B e indivíduos saudáveis.. In: Reunião Anual da Federação de Sociedades de Biologia Experimenta, 2009, Águas de Lindóia. Anais da FESBE, 2009., 2009.

20. **Souza, P.O.**; BOOIJ, H. G. ; LEVY, D. ; MACIEL, F.V.R. ; PEREIRA, J. ; BYDLOWSKI, S. P. . Polimorfismo da glutationa-S-transferase em pacientes com linfoma difuso de grandes células B. Relação com o tratamento e prognóstico.. In: Reunião Anual da Federação de Sociedades de Biologia Experimental, 2009, Águas de Lindóia. Anais da FESBE, 2009., 2009.

21. Vassão. R.C. ; Rombola, D.L.F. ; **Oliveira-Souza, P.** ; Costa, VA ; Maria, D.A. . Determination of Jararhagin Effects Over the Proliferative Activity and Cell Cicle Phases in Experimental Melanoma. In: XLI Congresso Da Sociedade Brasileira de Medina Tropical, 2005, Florianópolis. Determination of Jararhagin Effects Over the Proliferative Activity and Cell Cicle Phases in Experimental Melanoma, 2005.

22. **Oliveira-Souza, P.**; Lapetina, D.L. ; Maria, D.A. . Efeito Terapêutico Anti-tumoral da Metaloproteinase Tipo Disintegrina Jararagina no Melanoma B16F10. In: XX Reunião Anual da Federação de sociedades de Biologia Experimental-FeSBE, 2005, Águas de Lindóia. Efeito Terapêutico Anti-tumoral da Metaloproteinase Tipo Disintegrina Jararagina no Melanoma B16F10, 2005.

23. Vassão. R.C. ; **Oliveira-Souza, P.** ; Festa Neto, C ; Maria, D.A. . Effects of imiquimod treatment on experimental melanoma. In: 2º Simpósio de Imunologia, 2005, Botucatu. Effects os imiquimod treatment on experimental melanoma, 2005.

24. **Oliveira-Souza, P.**; Maria, D.A. . Avaliação Histopatológica e Anti-tumoral da Metaloproteinase-Tipo Disintegrina Jararagina no Melanoma B16F10. In: 13º Simpósio Internacional de Iniciação Científica da USP, 2005, Ribeirão Preto. Avaliação Histopatológica e Anti-tumoral da Metaloproteinase-Tipo Disintegrina Jararagina no Melanoma B16F10, 2005.

25. **Oliveira-Souza, P.**; Rodrigues, C.J. ; Ruiz, I.R.G. ; Maria, D.A. . Antitumorigenicity of jararhagin toxin in B16F10 murine melanoma cells. In: VII Reunião Científica Anual do Instituto Butantan, 2005, São Paulo. Antitumorigenicity of jararhagin toxin in B16F10 murine melanoma cells, 2005.

26. Costa, VA ; **Oliveira-Souza, P.** ; DAlmeida, V ; Roseiro, F ; Ribeiro, J.F. ; Bastos, S ; Quincoces, J ; Jasiulionis, M.G. . In vivo and in vitro Antitumoral Activity of Brazilian Propolis Derived Compounds. In: XXXIII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular - SBBq, 2004, Caxambú. In vivo and in vitro Antitumoral Activity of Brazilian Propolis Derived Compounds, 2004.

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Apresentações de Trabalho

1. **Oliveira-Souza, P.**; BOOIJ, H. G. ; LEVY, D. ; MACIEL, F.V.R. ; PEREIRA, J. ; BYDLOWSKI, S. P. . Polimorfismos da glutationa-S-Transferase (GSTM1, GSTT1 e GSTP1) em pacientes portadores de Linfoma Difuso de Grandes Células B. relação com o tratamento e prognóstico.. 2009. (Apresentação de Trabalho/Congresso).

2. **Souza, P.O.**; LEVY, D. ; MASELLI, L.M.F. ; MACIEL, F.V.R. ; PEREIRA, J. ; BYDLOWSKI, S. P. . Polimorfismo no gene MDR1 em pacientes com Linfoma Difuso de Grandes Células B e indivíduos saudáveis.. 2009. (Apresentação de Trabalho/Congresso).

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5. **Oliveira-Souza, P.**; LEVY, D. ; MACIEL, F.V.R. ; Maria, D.A. ; PEREIRA, J. ; BYDLOWSKI, S. P. . Características polimórficas do gene MDR1 em pacientes com linfoma difuso de grandes celulas B.. 2009. (Apresentação de Trabalho/Congresso).

6. **Oliveira-Souza, P.**; LEVY, D. ; BOOIJ, H. G. ; MACIEL, F.V.R. ; PEREIRA, J. ; BYDLOWSKI, S. P. . Association of genetic polymorphisms of glutatione-S-transferase genes and overall response/clinical features in diffuse large B-cell lymphoma patients treated with Rituximab.. 2009. (Apresentação de Trabalho/Congresso).

7. **Oliveira-Souza, P.**; BOOIJ, H. G. ; LEVY, D. ; MACIEL, F.V.R. ; BYDLOWSKI, S. P. . Polimorfismos do gene da glutationa-S-transferase (GSTM1, GSTT1 e GSTP1) em pacientes com linfoma difuso de grandes celulas B: Implicação terapêutica e de prognostico.. 2009. (Apresentação de Trabalho/Congresso).

Produção técnica

Processos ou técnicas

1. ★ Quincoces, J ; Rando, D.G. ; Maria, D.A. ; Martins, C.A.S. ; Pardi, P.C. ; **Oliveira-Souza, P.** . Métodos de obtenção de penta-1,4-dien-3-onas e de cicloexanonas substituídas e derivados com propriedades antitumorais e antiparasitárias, processo para preparar 1,5-bis-(aril)-penta-1,4-dien-3-onas alquiladas e aciladas, processo para preparar 4-nitro-3,5-diaril-cicloexanonas substituídas, processo para preparar 2,6-dibenzilideno-4-nitro-3,5-diaril-cicloexanonas substituídas, processo para prepar. 2006.

Patentes e registros

Patente

A Confirmação do status de um pedido de patentes poderá ser solicitada à Diretoria de Patentes (DIRPA) por meio de uma Certidão de atos relativos aos processos

1. Rando, D.G. ; Martins, C.A.S. ; Maria, D.A. ; **Souza, P.O.** ; SUÁREZ, J. A. P. Q. . Method to prepare sodium 4-[5-(4-hydroxy-3-methoxyphenyl)-3-oxo-penta-1,4-dienyl]-2-methoxy-phenolate, DM-1 and derivatives with antitumoral and antiparasitic activity.. 2006, Estados Unidos.
Patente: Privilégio de Inovação. Número do registro: 8859625, título: "Method to prepare sodium 4-[5-(4-hydroxy-3-methoxyphenyl)-3-oxo-penta-1,4-dienyl]-2-methoxy-phenolate, DM-1 and derivatives with antitumoral and antiparasitic activity." , Instituição de registro: United States Patent and Trademark Office, Depositante (s): José Augustin Pablo Quincoces Suárez;Universidade Bandeirante de São Paulo, Depósito: 06/07/2006; Depósito PCT: 06/07/2006. Instituição(ões) financiadora(s): FAPESP.

2. Maria, D.A. ; Rando, D.G. ; Martins, C.A.S. ; Pardi, P.C. ; **Souza, P.O.** ; SUÁREZ, J. A. P. Q. . Methods to prepare penta-1,4-dien-3-ones and substituted cyclohexanones and derivatives with antitumoral and antiparasitic properties, the compounds and their uses.. 2013, Japão.
Patente: Privilégio de Inovação. Número do registro: 5317290, título: "Methods to prepare penta-1,4-dien-3-ones and substituted cyclohexanones and derivatives with antitumoral and antiparasitic properties, the compounds and their uses." , Instituição de registro: Japanese Patent Office, Depositante (s): José Augustin Pablo Quincoces Suárez, Depósito: 19/07/2013; Depósito PCT: 19/07/2013. Instituição(ões) financiadora(s): FAPESP; Universidade Anhanguera de São Paulo.

3. SUÁREZ, J. A. P. Q. ; Maria, D.A. ; Rando, D.G. ; Martins, C.A.S. ; Pardi, P.C. ; **Souza, P.O.** . Method to prepare sodium 4-[5-(4-hydroxy-3-methoxyphenyl)-3-oxo-penta-1,4-dienyl]-2-methoxy-phenolate, DM-1 and derivatives with antitumoral and antiparasitic activity.. 2014, Alemanha.
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Eventos

Participação em eventos, congressos, exposições e feiras

1. 12 International Symposium on Lactic Acid Bacteria.Effect of grape pomace on Lactobacillus acidophilus and Streptococcus thermophylus viability and acidification kinetics. 2017. (Simpósio).
2. 16. BOKU Symposium Tierernährung.Discontinuous fermentation process by probiotic bacterium to produce antimicrobial peptide with potential applicatiion as food preservative.. 2017. (Simpósio).
3. 25 SIICUSP - Simpósio Internacional de Iniciação Científica e Tecnológica da Universidade de São Paulo.Otimização da produção de bacteriocina por Lactococcus lactis a partir de meio de cultivo suplementado com fontes de carbono e nitrogênio. 2017. (Simpósio).
4. 29 Congresso Brasileiro de Microbiologia. DISCONTINUOUS FERMENTATION PROCESS BY PROBIOTIC BACTERIUM TO PRODUCE ANTIMICROBIAL PEPTIDE WITH POTENTIAL APPLICATION AS FOOD PRESERVATIVE. 2017. (Congresso).
5. XV Simpósio de Biossegurança e Segurança Química e Biológica em Instituições de Ensino e Pesquisa. 2016. (Simpósio).
6. 28 Congresso Brasileiro de Biotecnologia. Efeito da inulina e sacarose no crescimento e produção de bacteriocina por Pediococcus pentosaceus. 2015. (Congresso).
7. 28 Congresso de Microbiologia. Cinética de acidificação e crescimento de Streptococcus thermofilus TA 040 e Lactococcus lactis CETC 4434 a partir de soro de leite. 2015. (Congresso).
8. L SUBFAB - 50 Semana Universitária Paulista de Farmácia. 2015. (Encontro).
9. Palestra Mechanistic studies in an "in vitro" model of the colon to study the role of thee gut microbiota in obesity. 2015. (Outra).
10. Palestra miRNAs e proteínas de união a RNA em neurogênese e tumores de cérebro. 2015. (Outra).
11. Palestra Prebiotics, Probiotics and Synbiotics and their Impact on Nutrients, Vitamins and Minerals; Instestinal microbiota in Synthesis ans Activation of Vitamin 12. 2015. (Outra).
12. Pharmaceutical Technology Seminar 2015 - New technologies and processes to to improve solubility of poorly soluble drugs - Ashland. 2015. (Seminário).
13. Pharmaceutical Technology Seminar - New technologies and processes to improve solubilityof poorly solubre drugs. 2015. (Seminário).
14. Workshop Scifinder. 2015. (Oficina).
15. XiV Simpósio de Biossegurança e Descarte de Produtos Químicos Perigosos e Organismos Geneticamente Modificados (OGMs) em Instituições de Ensino e Pesquisa. 2015. (Simpósio).
16. XX Semana Farmacêutica de Ciência e Tecnologia.Efeito da inulina e sacarose no crescimento e produção de bacteriocina por Pediococcus pentosaceus. 2015. (Simpósio).
17. XX Semana Farmacêutica de Ciência e Tecnologia.Efeito do pH e suplementação com polidextrose no crescimento e síntese de bioprodutos por Pediococcus pentosaceus ATCC 43200 durante crescimento em shaker. 2015. (Simpósio).
18. XX Semana Farmacêutica de Ciência e Tecnologia.Efeito de diferentes prebióticos no crescimento e produção de bacteriocina por Lactobacillus plantarum. 2015. (Simpósio).
19. 22 Simpósio Internacional de Iniciação Científica e Tecnológica da Ua USP - SIICUSP.Avaliadora da primeira etapa do SIICUSP 2014. 2014. (Simpósio).
20. American Society of Hematology Annual Meeting. Polimorfismos de FCgammaRIIa não estão associados com resposta completa no linfoma difuso de grandes células B tratados com Rituximabe. 2009. (Congresso).
21. American Society of Hematology Annual Meeting. Associação de polimorfismos genéticos de genes da glutationa-S-transferase e resposta completa/características clínicas em pacientes com linfoma difuso de grandes células B tratados com Rituximabe.. 2009. (Congresso).
22. Congresso Brasileiro de Hematologia e Hemoterapia, HEMO. Características polimórficas do gene MDR1 em pacientes com linfoma difuso de grandes celulas B.. 2009. (Congresso).
23. Congresso Brasileiro de Hematologia e Hemoterapia, HEMO. . Polimorfismos do gene da glutationa-S-transferase (GSTM1, GSTT1 e GSTP1) em pacientes com linfoma difuso de grandes celulas B: Implicação terapêutica e de prognostico.. 2009. (Congresso).
24. XXIV Reunião Anual da Federação de Sociedades de Biologia Experimental. Polimorfismo no gene MDR1 em pacientes com linfoma difuso de grandes células B e indivíduos saudáveis.. 2009. (Congresso).
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27. XXIV Reunião da Federação de Sociedades de Biologia Experimental - FESBE. Citocromo P450 e Glutationa-metil-transferases. Importância nas neoplasias.. 2009. (Congresso).

28. 13º Simpósio Internacional de Iniciação Científica da USP. 2005. (Simpósio).

29. 1º Curso de Redação Científica. 2005. (Outra).

30. Curso de Extensão Universitária. 2005. (Outra).

31. Curso de Radioproteção. 2005. (Outra).

32. V Curso de Técnicas de Biologia Molecular e Cultura de Células. 2005. (Outra).

33. VII Reunião Científica Anual do Instituto Butantan. 2005. (Encontro).

34. VII Reunião Científica Anual do Instituto Butantan. 2005. (Encontro).

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36. XLI Congresso da Sociedade Brasileira de Medicina Tropical. 2005. (Congresso).

37. XX Reunião Anual da Federação de Sociedades de Biologia Experimental-FeSBE. 2005. (Congresso).

38. 7º Encontro Regional de Biomedicina. 2004. (Encontro).

39. Curso Básico de Uveítes. 2004. (Outra).

40. Sociedade Brasileira de Bioquímica e Biologia Molecular. 2004. (Congresso).

41. XXVI Congresso Latinoamericano de Química. 2004. (Congresso).

42. 4º Simpósio de Biomedicina. 2003. (Simpósio).

43. 3º Simpósio de Biomedicina. 2002. (Simpósio).

44. Curso de Imunologia da Gestação: Aspectos Teóricos e Práticos. 2002. (Outra).