UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de São José do Rio Preto

Aline Teodoro de Paula

Leuconostoc mesenteroides SJRP55 isolada de mussarela de búfala: perfil probiótico e bacteriocinogênico

São José do Rio Preto
2013

Aline Teodoro de Paula

Leuconostoc mesenteroides SJRP55 isolada de mussarela de búfala: perfil probiótico e bacteriocinogênico

Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Microbiologia, junto ao Programa de Pós-Graduação em Microbiologia, Área de Concentração - Microbiologia Industrial, Ambiental e de Alimentos, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de São José do Rio Preto.

Orientador: Profª. Drª. Ana Lúcia Barretto Penna

São José do Rio Preto
2013

Aline Teodoro de Paula

Leuconostoc mesenteroides SJRP55 isolada de mussarela de búfala: perfil probiótico e bacteriocinogênico

Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Microbiologia, junto ao Programa de Pós-Graduação em Microbiologia, Área de Concentração - Microbiologia Industrial, Ambiental e de Alimentos, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de São José do Rio Preto.

Comissão Examinadora

Profª. Drª. Ana Lúcia Barretto Penna
UNESP – São José do Rio Preto
Orientador

Prof. Dr. Svetoslav Dimitrov Todorov
USP – São Paulo

Profª. Drª. Vanessa Bíscola
USP – São Paulo

Prof. Dr. Rafael Chacon Ruiz Martinez
USP – São Paulo

Profª. Drª. Mara Corrêa Lelles Nogueira
FAMERP – São José do Rio Preto

São José do Rio Preto
16 de dezembro de 2013

Paula, Aline Teodoro de.
Leuconostoc mesenteroides SJRP55 isolada de mussarela de búfala : perfil probiótico e bacteriocinogênico / Aline Teodoro de Paula. -- São José do Rio Preto, 2013
140 f. : il., tabs.

Orientador: Ana Lúcia Barretto Penna
Tese (doutorado) – Universidade Estadual Paulista "Júlio de Mesquita Filho", Instituto de Biociências, Letras e Ciências Exatas

1. Microbiologia industrial. 2. Leite fermentado - Microbiologia. 3. Probióticos. 4. Bacteriocinas. 5. Leuconostoc. I. Penna, Ana Lúcia Barretto. II. Universidade Estadual Paulista "Júlio de Mesquita Filho". Instituto de Biociências, Letras e Ciências Exatas. III. Título.

CDU – 637.146:576.8

Ficha catalográfica elaborada pela Biblioteca do IBILCE
UNESP - Campus de São José do Rio Preto

Um sonho que se sonha só é só um sonho que só. Um sonho que se sonha junto é realidade!

Raul Seixas

AGRADECIMENTOS

A Deus, por me auxiliar durante toda execução desse trabalho e pelo equilíbrio mantido durante os momentos difíceis que obtive durante esse trajeto.

À minha família, meus pais, Almir e Ligia e ao meu irmão, Anderson, pelo amor incondicional. Por acreditarem no meu sonho, na minha capacidade e determinação para atingir esse objetivo, ajudando sempre com boas palavras e vibrações positivas.

Ao meu noivo, Fernando, por estar sempre ao meu lado acreditando e incentivando na concretização desse sonho, pela paciência, amor, amizade e companheirismo.

As amigas do Laboratório de Leite e Derivados pelo carinho, companheirismo e amizade. Foram anos de conversas saudáveis, risos e muito aprendizado. Muitas já concluíram essa etapa e já traçam novos caminhos, mas nunca me esquecerei do quanto foram primordiais no meu crescimento pessoal, profissional e em grupo. Um grande abraço para vocês: Íris, Grazi, Raquel, Luanilda, Tatiana, Viviane, Ceci e Sabrininha.

Às minhas companheiras de trabalho de mestrado, Janaína e de doutorado Bia, pelos ensinamentos profissionais, pela tolerância, pela paciência e pelo carinho que sempre tiveram por mim, além de sempre acreditarem no nosso trabalho.

À professora e orientadora Ana Lúcia, nossa mãe científica e exemplo de mulher guerreira. Obrigada por confiar no meu trabalho, por acreditar na minha capacidade profissional, por me auxiliar e incentivar sempre quando foi preciso, e pela boa vivência profissional que tivemos durante esses cinco anos de trabalho.

Ao professor Svestoslav (Slavi), por todo auxilio e conhecimento profissional para execução desse trabalho.

À professora Bernadette, por me receber em seu laboratório para o desenvolvimento de parte desse trabalho.

Às minhas amigas-irmãs do meu lar Rio Pretense: Tamara, Rubia e Talita, pela paciência, carinho, companheirismo e amizade durante todo o tempo que vivemos juntas.

Aos meus novos amigos "franco-brasileiros" Vanessa e Fabrício, pelos ensinamentos laboratoriais, pelo companheirismo, amizade e apoio durante todo o momento que vivi na França.

Ao grupo de pesquisa Francês: Professores Xavier, Jean-Marc e Thomas e ao técnico Yvan pela oportunidade de me aceitarem como aluna e pelos ensinamentos e auxílios prestados na execução de parte desse trabalho.

Aos técnicos Ginaldo e Luiz, pelos auxílios prestados.

À FAPESP pelo suporte financeiro para execução desse projeto.

À CAPES pelo fornecimento das bolsas de doutorado e doutorado sanduíche.

Ao Programa de Pós-graduação em Microbiologia da UNESP/IBILCE, principalmente as professoras Paula e Eleni, pelos ensinamentos científicos e profissionais transpassados, além de ser um exemplo de profissional a serem seguidas.

SUMÁRIO

LISTA DE FIGURAS

Página

Capítulo II

gray bar), final population (dark gray bar), and final population in presence of CFS (black bar). Present results are average of the 3 independent repetitions and SD is not indicated.

F**igure 4** Reversed phase chromatography profile of active peptides produced by *Leuconostoc mesenteroides* SJRP55 (peaks 1 and 2) on an analytical RP Nucleosil C_8 (Symmetry 300 Å C_8, 5 µm, 7.8 x 100 mm) equilibrated with solvent A (0.5% solvent B, 95% MilliQ water, 0.03% TFA). Elution was performed at a flow-rate of 2.5 ml/min with a linear gradient from 0 to 60% solvent B (80% acetonitrile, 20% isopropanol and 0.05% TFA) for 30 min. 109

Figure 5 Mass spectrometry (ESI-MS-MS) of mesentericin W-SJRP55 and mesentericin Z-SJRP55. 111

LISTA DE TABELAS

RESUMO

As bactérias acidoláticas formam um grupo de grande importância para as indústrias de alimentos, sendo comumente utilizadas em produtos fermentados. *Leuconostoc mesenteroides* pode apresentar propriedades tecnológicas interessantes para aplicação em alimentos, tais como o potencial probiótico e a produção de bacteriocinas. O objetivo deste estudo foi avaliar o perfil probiótico de *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 e aplicá-lo em leites fermentados, além de caracterizar, purificar e identificar as bacteriocinas produzidas por esse micro-organismo. Na avaliação do potencial probiótico e da viabilidade na presença de medicamentos, a cultura mostrou resistência às condições de estresse (ácido e sais biliares) e também foi capaz de desconjugar sais biliares. A tolerância ao NaCl foi dependente da temperatura, assim como a capacidade de agregação. *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 mostrou boas propriedades de adesão (co-aggregação, hidrofobicidade, aderência às células Caco-2), presença da enzima β–galactosidase e sensibilidade à maioria dos antibióticos testados. No entanto, alguns medicamentos analgésicos inibiram o desenvolvimento desta cepa. *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 em co-cultura com *Streptococcus thermophilus* TA040 apresentou viabilidade durante o período de estocagem dos leites fermentados. Na avaliação da produção de substâncias antimicrobianas, *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 produziu compostos com ampla inibição sobre cepas de *Listeria* spp., mas não para espécies de *Lactobacillus* spp. Os compostos antimicrobianos foram estáveis em altas temperaturas, em baixos valores de pH, e na presença de compostos químicos, foram sensíveis a enzimas proteolíticas e resistentes a α-amilase, lipase e catalase. A temperatura ótima de produção do composto foi 25°C. As substâncias antimicrobianas apresentaram um leve efeito bactericida sobre diferentes cepas de *Listeria innocua* e *Listeria monocytogenes* e reduziram em um ciclo log as células viáveis de *Lc. mesenteroides.* O composto não demonstrou estar adsorvido às células produtoras. Os peptídeos ativos produzidos foram purificados pela precipitação em sulfato de amônio, em coluna de afinidade e por cromatografia líquida de fase reversa. A espectrometria de massa e a análise dos aminoácidos confirmaram que as bacteriocinas produzidas pela cepa são idênticas à mesentericina Y105 e B105. Foi observada a presença dos genes de virulência e resistência: proteínas de superfície de *Enterococcus* (esp), adesão ao colágeno (ace), vancomicina (vanA) e a ausência dos genes produtores de aminas biogênicas. Os resultados obtidos mostraram o potencial probiótico *in vitro* da cultura *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 e a aplicação promissora como cultura para bioconservação de leites fermentados. As bacteriocinas purificadas também podem ser aplicadas como uma barreira tecnológica para a conservação de alimentos. Palavras-chave: potencial probiótico, bacteriocinas, mesentericina Y105 e mesentericina B105, leite fermentado.

ABSTRACT

Lactic acid bacteria are an important group for food industry, being commonly used in food fermentation. Leuconostoc mesenteroides can show interesting technological properties in food application, such as probiotic potential and bacteriocins production. The objective of this study was to evaluate the probiotic potential of Lc. mesenteroides subsp. mesenteroides SJRP55 and its application in fermented milk, and to characterize, purify and identify the bacteriocins produced by this microorganism. In the evaluation of probiotic potential and viability in presence of medicaments, the culture showed resistance to stress conditions (acid and bile salts) and it was also able to deconjugate bile salts. The tolerance to NaCl was temperature dependent, as well as the results obtained for the aggregation assays. Lc. mesenteroides subsp. *mesenteroides* SJRP55 *presented good adhesion properties (co-aggregation, cell surface hydrophobicity and adherence to Caco-2 cells), demonstrated β–galactosidase activity and it was sensitive to most of the tested antibiotics. However, some analgesic drugs inhibited the growth of this strain. Lc. mesenteroides subsp. mesenteroides SJRP55, in co-culture with Streptococcus thermophilus* TA040*, showed viability in fermented milk during the storage conditions. During the evaluation of production of antimicrobial substances, Lc. mesenteroides subsp. mesenteroides SJRP55 produced antibacterial compounds with strong inhibition against Listeria spp. strains and no inhibition against Lactobacillus spp. The antimicrobial substances were stable to high temperatures, low pH values, in presence of chemical compounds, but sensitive to proteolytic enzymes and resistant to α-amylase, lipase and catalase enzymes. The optimal temperature for antimicrobial compounds production was 25°C. The compounds showed slight bactericide effect against Listeria innocua and Listeria monocytogenes strains and reduced one log cycle of viable cells of Lc. mesenteroides. No adsorption to the producer cells was found. The active compounds were purified by ammonium sulphate precipitation, affinity column and reverse-phase chromatography. Mass spectrometry and aminoacids analyses showed that the bacteriocins produced by Lc. mesenteroides subsp. mesenteroides SJRP55 were identical to mesentericin Y105 and B105. The strain presented positive results for some genes encoding virulence factors, such as enterococcal surface protein (esp), collagen adhesion (ace) and intrinsic vancomycin resistance (vanA); however, biogenic amines encoding genes were not observed. The results showed the in vitro probiotic potential of Lc. mesenteroides subsp. mesenteroides SJRP55 suggested and its application as a promisor biopreservative culture in fermented milk. The purified bacteriocins can also be applied as a hurdle technology in food preservation.*

Keywords: *Probiotic potential, bacteriocins, mesentericin Y105 and mesentericin B105, fermented milk.*

INTRODUÇÃO E JUSTIFICATIVA

Nos últimos anos, tem aumentado o interesse dos consumidores por produtos naturais, menos processados, livre de conservantes químicos e que proporcionam benefícios à saúde. Os produtos lácteos, fermentados por bactérias acidoláticas (BAL) podem atender a estas características.

Diversas bactérias acidoláticas podem atuar como probióticas, ou seja, quando administradas em quantidade adequada, conferem benefícios à saúde do consumidor (FAO/WHO 2002), tais como: equilíbrio da microbiota intestinal, aumento da resposta imune, redução da intolerância à lactose, inibição de micro-organismos patogênicos pela produção de compostos naturais, entre outros benefícios (Reis et al., 2011; Amara e Shibl, 2013).

Por outro lado, os produtos lácteos, por possuírem elevado valor nutritivo e alta atividade de água, podem propiciar o desenvolvimento de micro-organismos patogênicos e deteriorantes. A presença desses micro-organismos pode ser consequência de falhas higiênico-sanitárias em diferentes etapas, que incluem entre a manipulação da matéria-prima, processamento e obtenção do produto final. Apesar dos produtos lácteos fermentados apresentarem características intrínsecas desfavoráveis (baixo valor de pH, elevada concentração de ácido lático e outros compostos orgânicos, baixas temperaturas de armazenamento e de concentração de oxigênio) para o desenvolvimento de diversos micro-organismos, muitos conseguem se desenvolver e promover modificações indesejáveis nesses produtos, e consequentemente, comprometem a vida de prateleira do produto e a saúde dos consumidores (Robinson, Tamime e Wszolek, 2002).

Durante o processo fermentativo, as BAL podem produzir compostos orgânicos, tais como: ácido lático, ácido acético, ácido propiônico, ácido fórmico, diacetil, acetaldeído, peróxido de hidrogênio, dióxido de carbono, bacteriocinas, além de outros compostos (De Vuyst e Leroy, 2007; Tharmaraj e Shah, 2009). As bacteriocinas são peptídeos microbianos sintetizados ribossomalmente (Cotter, Hill e Ross, 2005), as quais podem atuar como bioconservantes em alimentos e/ou inibir espécies patogênicas presentes no intestino. Várias espécies de BAL, isoladas de diferentes matrizes (trato gastrointestinal e urogenital de humanos e animais, produtos fermentados, carnes, pescados, frutas e vegetais), podem produzir substâncias com atividade antimicrobiana sobre diversos grupos de micro-organismos deteriorantes e patogênicos Gram-positivos (*Listeria* sp., *Clostridium* sp., *Staphylococcus aureus*, *Bacillus* sp.), Gram-negativos (*Salmonella* Typhimurium, *Helicobacter pylori*, *Campylobacter jejuni*, *Escherichia coli*), algumas espécies de fungos e vírus (Svetoch e Stern, 2010; Adebayo e Aderiye, 2010; Stoyanova et al., 2010; Todorov et al., 2010; Lagenaur et al., 2011).

Diversos estudos têm se dedicado em isolar bactérias acidoláticas do seu ambiente natural, com o objetivo de investigar o potencial probiótico desses micro-organismos, e também sua capacidade de produzir bacteriocinas tão efetivas quanto a nisina, uma bacteriocina comercial e reconhecida amplamente como segura pela *Food and Agriculture Organization/World Health Organization* (FAO/WHO), *Food and Drug Administration* (FDA) e União Europeia (1983). Estudos demonstram o potencial probiótico e a produção de bactericionas por diversas espécies de *Leuconostoc mesenteroides* (Chang et al., 2010; Seo et al., 2012; Ryu e Chang, 2013).

Leuconostoc mesenteroides são bactérias Gram-positivas, catalase negativas, com baixo conteúdo (31–49%) de guanina + citosina (G + C) na sequência do DNA ribossomal, heterofermentativas (fermentando glucose em D-ácido lático, etanol/ ácido acético e CO_2, utilizando a via fosfocetolase), apresentam morfologia cocóides ou cocobacilos, são imóveis, fastidiosas, não formadoras de esporos, anaeróbias facultativas, mesofílicas, não produzem amônia a partir da arginina, podem sobreviver em até 7% NaCl, e apresentam desenvolvimento ótimo entre 20 a 30^{o}C. As espécies pertencentes a este gênero podem ser encontradas em diversos tipos de ambiente: vegetais, cereais, silagem, frutas, vinho, peixe, carne e produtos lácteos (Sneath, 1986; Stiles e Holzapfel, 1997; Ray, 2004; Mäki, 2004; Hemme e Foucaud-Scheunemann, 2004; Trias et al., 2008; Xiraphi et al., 2008; Albesharat et al., 2011; Giraffa et al., 2012).

Assim, o estudo de novas cepas produtoras de bacteriocinas com o potencial de atuar como bioconservante e com potencial probiótico é de grande interesse para a aplicação tecnológica industrial e na área clínica. Os conhecimentos gerados poderão resultar na aplicação destas BAL em processos biotecnológicos inovadores.

OBJETIVOS

Com base nas evidências citadas, este trabalho teve como objetivo geral:

- Avaliar o potencial probiótico de *Leuconostoc mesenteroides* subsp. *mesenteroides* SJRP55 e sua aplicação em leites fermentados, além de caracterizar, purificar e identificar as bacteriocinas produzidas por esta cepa.

O presente trabalho foi organizado em quatro capítulos para melhor distribuição e entendimento dos assuntos abordados. O Capítulo I consiste em uma Revisão de Literatura sobre tema abordado nessa tese, elaborado na forma de artigo de revisão. Os capítulos II e III foram redigidos na forma de artigos científicos. Estes artigos serão submetidos à publicação em revistas internacionais. O Capítulo IV apresenta as conclusões gerais desse trabalho.

Os objetivos específicos dos capítulos foram:

Capítulo I

- Revisar o potencial probiótico e bacteriocinogênico de *Leuconotoc mesenteroides*, assim como investigar os casos de deterioração e patogenicidade relacionados a essa espécie.

Capítulo II

- Avaliar o potencial probiótico de *Leuconotoc mesenteroides* subsp. *mesenteroides* SJRP55 (tolerância em diferentes valores de pH, concentrações de sais biliares e NaCl; desconjugação dos sais biliares; capacidade de auto- e co-agregação; hidrofobicidade da superfície celular; adesão a células Caco-2; presença de diferentes enzimas; resistência a diferentes antibióticos e viabilidade na presença de medicamentos), além da aplicação em leites fermentados.

Capítulo III

- Avaliar a natureza do composto inibidor produzido por *Leuconotoc mesenteroides* subsp. *mesenteroides* SJRP55 (espectro de inibição sobre diversos micro-organismos indicadores; efeito do composto antimicrobiano na presença de enzimas, de diferentes valores de pH, da temperatura e de agentes químicos; cinética de produção do composto e modo de ação sobre diferentes micro-organismos indicadores; adsorção do composto antimicrobiano na superfície celular; purificação e identificação das bacteriocinas produzidas pela cepa; identificação dos genes produtores das bacteriocinas, e presença de genes de virulência, resistência a antibióticos e aminas biogênicas).

Capítulo IV

- Elaborar a conclusão geral sobre o trabalho.

Capítulo I

The two faces of *Leuconostoc mesenteroides* in food systems

Este Capítulo será submetido à Revista *Food Control*

The two faces of *Leuconostoc mesenteroides* in food systems

Aline T. de Paula[a], Ana Beatriz Jeronymo-Ceneviva[a], Svetoslav D. Todorov[b], Ana Lúcia B. Penna[a*]

[a] UNESP - Sao Paulo State University, Department of Food Engineering and Technology, Rua Cristovão Colombo, 2265, 15.054-000, São José do Rio Preto, Brazil Sao Jose do Rio Preto, Brazil

[b] USP - Sao Paulo University, Faculty of Pharmaceutical Sciences, Department of Food Science and Experimental Nutrition, Av. Prof. Lineu Prestes, 580, bl. 13B, 05508-000, Sao Paulo, Brazil

*Corresponding author: Ana Lúcia Barretto Penna, UNESP - Sao Paulo State University, Food Engineering and Technology Department, Rua Cristovão Colombo, 2265, 15.054-000, +55 17 3221-2266, São José do Rio Preto, Brazil. E-mail: analucia@ibilce.unesp.br

Abstract

Leuconostoc mesenteroides is a lactic acid bacterium (LAB) that has been isolated from different sources. Some of its strains are able to produce bacteriocins, and most of them belongs to class IIa and shows anti-*Listeria* activity. *Lc. mesenteroides* specie can be a probiotic candidate. Promising studies have shown the potential of this specie to survive and grow under different stress conditions present in the gastrointestinal tract (GIT). On the other hand, this microorganism can cause spoilage in some types of food matrices. Scarce reports have shown the pathogenic potential of this specie. In this review, we focus on an overview of *Lc. mesenteroides* bacteriocins-producing strains and its probiotic potential, in contrast with the spoilage and pathogenic cases reported in the literature.

Keywords: bacteriocins, biopreservation, probiotic, spoilage and pathogenic.

1. Introduction

Leuconostoc genus belongs to Firmicutes phylum, which includes Gram positive, heterofermentative (fermenting glucose to D-lactic acid, ethanol/acetic acid, and CO_2 from phosphoketolase pathway) microorganisms, and presents coccoid or rod-like shape. These bacteria are usually presented in pairs or short chains, non-motile, non-spore forming, catalase negative, anaerobic facultative, mesophilic and do not produce ammonia from arginine. It displays complex nutritional requirements, including different amino acids. Furthermore, it exhibits absence of H_2S formation, growth in presence of 7% NaCl and low-GC content (31–49%). This microorganism has mesophilic characteristic with the optimal growth between 20 and 30 ^{o}C. Species belonging to genus *Leuconostoc* can be found mainly in vegetables, cereals, silage, fruits, wine, fish, meat and dairy products (Stiles & Holzapfel, 1997; Ray 2004; Mäki 2004; Hemme & Foucaud-Scheunemann, 2004; Trias, Badosa, Montesinos & Bañeras, 2008; Xiraphi et al., 2008; Albesharat, Ehrmann, Korakli, Yazaji & Vogel, 2011; Giraffa, 2012). A significant review published by Hemme and Foucaud-Scheunemann (2004) presents an overview about *Leuconostoc* species, their habitat, taxonomy, metabolism and genetics, their implications in health and safety, and their potential use in dairy technology and functional foods.

Only a few reports about probiotic potential and bacteriocin applications using *Lc. mesenteroides* species have been found in the literature. Moreover, *Leuconostoc* spp. has a promisor future for application in dairy foods as bacteriocinogenic and/or probiotic microorganism through enhance of physicochemical characteristics of these products, mainly in cheeses. In this paper we performed an overview of *Lc. mesenteroides* potential for application as a probiotic and bacteriocin-producing culture, in contrast with its relationship to spoilage process and pathogenic cases reported in the literature.

2. Probiotic and bacteriocinogenic potential

Probiotics are defined as live non-pathogenic microorganisms that, when administrated in adequate amounts confer a health benefit to the consumer (FAO/WHO, 2002). The candidate strain to be considered as a probiotic culture should be able to survive under the stressful conditions of the gastrointestinal tract ; be able to compete, adhere and colonize the intestine cells; present therapeutic benefits; be safe for application in food (be non-pathogenic, do not present virulence genes nor antibiotics resistance and be genetically stable) and with adequate technological features (viable during the storage period, bacteriophage resistance, production in large scale). All of these properties should be validated and documented and the replicate results, found by *in vitro* trials, should be confirmed by *in vivo* assays (FAO/WHO, 2002; Reis, Casarotti, Paula, & Penna, 2011; Fontana, Bermudez-Brito, Plaza-Diaz, Muñoz-Quezada, & Gil, 2013). There are a remarkable number of reports in the literature that describes several LAB strains as potential probiotic cultures. However, several researches limited their studies in the *in vitro* assays because the list of required for a strain criteria to be considered as a probiotic is extensive and strict. Giraffa (2012) described a general overview of main criteria and methods that showed be considered important in the selection process of probiotic strains. Multiple studies describe countless benefits of probiotic cultures, such as increase in the host's immune response, alleviation of symptoms of lactose intolerance, production of some vitamins, usefulness in the treatment of many types of diarrhea, competition and inhibition of pathogenic microorganisms, by production of antibacterial components in GIT (e.g. bacteriocins, lactic acid, hydrogen peroxide and others), decrease of cholesterol levels, reduction of risk of colon cancer and alleviation of allergic symptoms, suppression of the gastric infection caused by *Helicobacter pylori*, improvement of oral health, influence on the course of critically ill patients and others (Adams, 2010; Singh, Kallali, Kumar, & Thaker, 2011; Reis, Casarotti, Paula, & Penna, 2011; Amara & Shibl, 2013; De Azevedo et al., 2013). However, some of these mechanisms, by which probiotics strains exert beneficial effects are largely unknown, not clearly understood or proved. In additional, some information about infection control, disease

treatment, health benefits, and the characterization of antimicrobial compounds produced by LAB can be obtained in the articles published by Amara and Shibl (2013) and Reis, Paula, Casarotti, and Penna (2012), respectively.

Bacteriocins are an abundant and diverse group of ribosomally synthesized antimicrobial peptides or proteins produced by *Bacteria* and *Archaea* and can be used as biopreservatives in food industry or be produced by LAB in the GIT, as a competitive factor (Dobson, Cotter, Ross, & Hill, 2012). The production of these compounds by these microorganisms, which is concern about to protect food against contamination and/or prevent the growth of foodborne pathogens and spoilage microorganisms have been attracted the attention of these companies (Cotter, Hill, & Ross, 2005). Moreover, the increase demand of consumers by 'bioproducts' with the addition of less chemical preservatives are drawing the attention of food industries, which are searching new biopreservatives compounds, such as bacteriocins. In addition, the resistance to high temperatures, low pH, the presence of organic solvents together with the fact that they are easily digested by human proteolytic enzymes make them an interesting tool for application in food preservation (Zouhir, Hammami, Fliss, & Hamidaet, 2010; Masuda et al., 2011). However, since the discovery of nisin, which is the only bacteriocin approved by Food and Agriculture Organization/World Health Organization (FAO/WHO), US Food and Drug Agency (FDA) and European Union (1983), the search of new successful bacteriocins, produced by LAB, are still labored-intensive.

Generally, bacteriocins are small cationic and amphipathic molecules, presenting α-helix and/or β-sheet protein structure, which can aggregate when present in liquid media or in high concentrations (Heng, Wescombe, Burton, Jack, & Tagg, 2007). Bacteriocins production is very usual for LAB. Besides, some characteristics seem to be common, such as (1) innate immunity to their own bacteriocin, (2) resistance or sensitiveness to others bacteriocins, (3) production of more than one antimicrobial compound, (4) the same bacteriocin can be produced by different genus, and (5) same subspecies can produce different compounds (Cotter, Hill, & Ross, 2005; Heng, Wescombe, Burton, Jack, & Tagg, 2007). Details about the biology of bacteriocins, their resistance, the molecular biology and their applications were summarized by

Cotter, Hill, and Ross (2005). In addition, the book "*Bacteriocins ecology and evolution*" edited by Riley and Chavan (2007), cover an extensive review about the diversity of bacteriocins produced by Gram-positive and Gram-negative bacteria.

Bacteriocins are classified based on their structure, molecular weight and mode of action (Cotter, Hill, & Ross, 2005; Heng & Tagg, 2006). Different classifications of bacteriocins have been proposed. One of the most accepted classifications was proposed by Klaenhammer (1993), which divides bacteriocins in four classes: (I) lanthionine-containing bacteriocins, (II) non-lanthionine-containing bacteriocins, (III) large heat-labile bacteriocins, (IV) complex moiety bacteriocins in combination with lipids and carbohydrates. In addition, this classification was reformulated by Cotter, Hill, and Ross (2005) in two categories: (I) lanthionine-containing bacteriocins, (II) non-lanthionine-containing bacteriocins and bacteriolysins. Although bacteriocin classification being still under discussion by several authors and continuous changing over time, the most consolidate and acceptable classification for bacteriocin is that one proposed by these last authors. Most of bacteriocins produced by *Leuconostoc* belong to class II, which are small (<10 kDa), heat-stable, non-lanthionine-containing bacteriocins with no post-translational modification (Klaenhammer, 1993). As mode of action, these peptides present an amphiphilic helical structure, which allows them to be inserted into the membrane, lead to depolarization, leakage of molecules and cell death (Cotter, Hill, & Ross, 2005). Moreover, the bacteriocins belonging to this group own an N-terminal region commonly called as 'pediocin-box'. This region is conserved among bacteriocins and presents a consensus sequence (YGNGVXaaC), which is responsible for the anti-*Listeria* activity. The C-terminal domains are less conserved and thought to determine the non-listerial antimicrobial spectrum (Cotter, Hill, & Ross, 2005). Different studies have reported the characteristics of class II bacteriocins; however, an overview published by Kjos (2011) highlighted some important recent progresses in the target recognition, resistance, immunity and genome of the non-lantibiotic (class II) bacteriocins. This review introduces an overview of *Lc. mesenteroides* as a bacteriocin producer and a potential probiotic strain.

The literature also describes the bacteriocin production (Limonet, Revol-Junelles, Cailliez-Grimal, & Millière, 2004; Jasniewski, Cailliez-Grimal, Younsi, Millière, & Revol-Junelles, 2008) and potential probiotic of *Leuconostoc* species (Chang, Shim, Cha, & Chee, 2010; Allameh, Daud, Yusoff, Saad, & Ideris, 2012). However, some of these studies are too preliminary.

3. Studies with *Lc. mesenteroides* as a potential probiotic strain

3.1 Resistance to gastric acidity and bile salts

The ability to tolerate and survive under the acid stomach environment and the resistance to bile salts found into the small intestine, is one criteria for the characterization of microorganism as a potential probiotic strain. According to the literature, different methodologies were used to evaluate the potential probiotic strains. Different isolates of *Lc. mesenteroides* have demonstrated the ability to survive at low pH: strain H4 and H5 from seafood products (Cho & Do, 2006); strain B-1 from natural yogurt (Vélez et al., 2007); strain PLsr-$1_{(w)}$ from whey (Rani & Agrawal, 2008); strains KFRI818, KFRI821, LA89406; strain YML003; and strains PH1, DM1 (Chang, Shim, Cha, & Chee, 2010; Seo et al., 2012; Ryu & Chang, 2013) all of them isolated from *kimchi*; strain CLFP 68 and strain LAB-4 both of them isolated from fish intestine (Pérez-Sánchez et al., 2011; Allameh, Daud, Yusoff, Saad, & Ideris, 2012); strain Lnm-1RM3 from *narezushi* (Nakamura et al., 2012). On the other hand, *Lc. mesenteroides* IM082 did not survive after 60 min in stomach reactor of a system that simulates the human upper GIT (Mainville, Arcand, & Farnworth, 2005). An interesting report showed the important role of cell membrane fatty acids in response to the pH reduction. An increase of unsaturated fatty acids such as palmitoleic acid (9.1%), oleic acid (5.8%), linoeic acid (5.4%) and linolenic acid (2.8%) was found at pH 3.0 (Rani & Agrawal, 2008).

Bile is produced in the liver, concentrated in the gall bladder and released into the duodenum, where it aids fat digestion by emulsifying and solubilizing lipids (Van Rennen & Dicks, 2011). The physiological concentration level of human bile varies and depends on race,

physiological conditions, and gender. Moreover, bile is an antimicrobial substance, which is constituted by bile acids, cholesterol and phospholipids. Studies suggest that all probiotic strains should be able to grow and survive in the presence of up to 0.3% of bile salts (Divya, Varsha, & Nampoothiri, 2012). Some studies have shown that strains of *Lc. mesenteroides* can survive under different concentrations of bile salts (Mainville, Arcand, & Farnworth, 2005; Cho & Do, 2006; Rani & Agrawal, 2008; Chang, Shim, Cha, & Chee, 2010; Seo et al., 2012; Nakamura et al., 2012; Ryu & Chang, 2013). However, this substance limited the growth of some *Lc. mesenteroides* subsp. *mesenteroides* strains (Allameh, Daud, Yusoff, Saad, & Ideris, 2012; Todorov & Dicks, 2008).

3.2 Bile salt hydrolase activity

Some LAB have the ability to hydrolysate bile salts by enzymes. These enzymes detoxify bile by deconjugating bile acids (Begley, Hill, & Gahan, 2006). Studies have shown that this phenomenon promoted by LAB increases the demand for cholesterol which, in turn, prompts the synthesis of more bile salts in the liver. This process may lead to a reduction of serum cholesterol level; however, researchers have not yet established the dose-effect relationship (Kumar, Kumar, Ghosh, & Ganguli, 2013). Nonetheless, to the best of our knowledge, there are currently no published studies on the ability of *Lc. mesenteroides* to deconjugate bile salts.

3.3 Adherence to mucus and/or human epithelial cells and cell lines

An important condition for LAB to colonize and survive in the GIT is its ability to adhere to mucus and the intestinal epithelial cells (Xu, Jeong, Lee, & Ahn, 2009). The cell surface of LAB is constituted by proteins, polysaccharides, and lipoteichoic acid, which can be responsible for the attachment of bacteria to different surfaces. Furthermore, the mechanisms of adherence to an epithelial surface involve both receptor-specific binding, charge and hydrophobic interaction (Ljungh & Wadström, 2006). Once attached to this system, the microorganisms can promote health benefits to the host, enhance the stimulation of human

immune system, compete for nutrients and produce antimicrobial compounds, which can interact positively or negatively with other microorganisms present in the GIT. The adhesion ability of probiotic strains has been studied using the *in vitro* model systems. However, different factors of the *in vitro* tests (food matrix, shelf-life, temperature, growth media, oxygen, pH, different types of cell lines) can modify the physicochemical properties of the bacterial surfaces and, consequently, their adhesion to the intestinal mucosa (Deepika, Rastall, & Charalampopoulos, 2011; Ryu & Chang, 2013). Moreover, the *in vitro* results should be interpreted with caution because the ability to adhere does not necessarily mean *in vivo* adhesion.

Adhesion of microorganisms is generally tested with mono-layers intestinal culture cells. The mucus layer works as a barrier or can provide the adhesion of the microorganism (Ouwehand, Kirjavainen, Grönlund, Isolauri, & Salminen, 1999). The mucus gel (gel covering the intestinal epithelial surface) is also used for adhesion studies. The mucus layer is the first contact between ingested bacteria and intestinal mucous membrane. Therefore, the results obtained with mucus adhesion assay can provide important additional data on probiotic adhesion properties and supplement the data obtained with cell lines without mucus-secreting ability (Caco-2 and HT-29). These cells lines can differentiate into enterocytes and can thus be used as a model for the small intestine epithelium. However, the bacterium adhesion is strain dependent and the ability to adhere in Caco-2 and HT-29 does not mean the adhesion to mucus gel (Saarela, Mogensen, Fondén, Mättö, & Mattila-Sandholm, 2000). Xu, Jeong, Lee, and Ahn (2009) suggested that *in vitro* adhesion to Caco-2 cells is correlated with competitive inhibition for attachment sites, which can competitively exclude foodborne pathogens. According to Russo et al. (2012) and Ryu and Chang (2013), bacterial adhesion increases with exopolysaccharides (EPS) synthesis, which is commonly produced by *Lc. mesenteroides* species (Hemme & Foucaud-Scheunemann, 2004; Nieto-Arribas, Seseña, Poveda, Palop, & Cabezas, 2010; Bendimerad, Kihal, & Berthier, 2012).

In most of time, the *in vitro* adhesion studies can be poor and not very well understood. Moreover, the *in vivo* trials are very limited due to ethical considerations, laborious techniques

and lack of volunteers. Several studies showed the adhesion of *Lc. mesenteroides* strains to cell lines (Caco-2 and/or HT-29 cells); strain B-1 isolated from yogurts (Vélez et al., 2007); strain CLFP68 isolated from intestine of rainbow trout (Pérez-Sánchez et al., 2011); and strains DM1 and PH1 isolated from *kimchi* (Ryu & Chang, 2013). In addition, strain PLsr-1(w) showed strong adherence to ileal epithelial cells of rats (Rani & Agrawal, 2008).

Hydrophobicity is another physicochemical property, which can facilitate the first contact between microorganisms and host cells (Shobharani & Agrawal, 2011). The bacterial cell surface contains proteins and polysaccharides with hydrophobic and hydrophilic properties (carboxylic groups and Lewis acid–base interactions), which is responsible for the adhesion of bacteria to surfaces (Van Der Mei & Busscher, 1998; Xu, Jeong, Lee, & Ahn, 2009). Moreover, the cell surface hydrophobicity is strain-specific and the presence of different nutrients or carrier food matrix may influence the expression of adhesion genes in the microorganisms (Ouwehand & Vesterlund, 2004; Raghavendra & Halami, 2009). The hydrophobicity of *Lc. mesenteroides* CLFP68 (11.42%) was significantly lower ($P < 0.05$) than the one observed for *Lactobacillus plantarum* (24.99%) and *Lactococcus lactis* (31.59%) (Pérez-Sánchez et al., 2011). The ability to adhere with different hydrocarbons provides the evidence that *Lc. mesenteroides* PLsr-$1_{(w)}$ strain can adhere and colonize the intestinal tract. Furthermore, sodium docecyl sulfate electrophoresis analysis of its surface protein showed a prominent protein band of 42 kDa, which is the S-layer protein responsible for the adhesion of the culture (Rani & Agrawal, 2008). *Lc. mesenteroides* 2M isolated from skin of healthy frogs exhibited a higher degree of hydrophobicity and contained basic components in the cell surface, according to their adhesion to chloroform than other LAB strains isolated from water and balanced feed. The results can indicate that these characteristics could be associated with the interaction with components of mucosal surfaces, such as epithelial cells and mucus that contain acid glycoconjugates (Pasteris, Roig Babot, Otero, Bühler, & Nader-Macías, 2009).

Other adhesion property frequently evaluated is the aggregation and co-aggregation capability, which can help probiotic cultures to adhere to the oral cavity, GIT, urogenital tract and modulate the immune system in the GIT (Ouwehand, Kirjavainen, Grönlund, Isolauri, &

Salminen, 1999; Divya, Varsha, & Nampoothiri, 2012). The co-aggregation ability can help to prevent colonization by invading foodborne pathogens (Xu, Jeong, Lee, & Ahn, 2009). Moreover, co-aggregation has been related to the ability to closely interact with pathogens, produce antimicrobial substances, inhibit the growth of these microorganisms in the GIT and block the dissemination of pathogens to other attachment sites (Collado, Surono, Meriluoto, & Salminen, 2007).

3.4 Metabolic activity

The metabolic activity is an important subject to study due the production of significant metabolites by microorganims. *Lc. mesenteroides* subsp. *mesenteroides* showed the ability to produce high concentrations of conjugated linoleic acid (Abd El-Salam et al., 2010), which has shown numerous health benefits, including antiatherogenic, antidiabetic, anti-inflammatory and anticarcinogenic properties. The introduction of probiotic strains (*Lc. mesenteroides* MTCC 5442 and *Bacillus subtilis* natto RG4365) in the probiotic *ragi malt* (functional food) resulted in the presence of high amounts of beneficial fatty acids like linoleic and linolenic acid, and increased the mineral content (iron and zinc) of the product (Vidyalaxme, Rovetto, Grau, & Agrawal, 2012).

Another health issue that could be positively influenced by probiotic cultures metabolism is the lactose intolerance. Some consumers of dairy products are lactose intolerant due the absence or low production amounts of β–galactosidase enzyme which causes discomfort after milk digestion (Raghavendra & Halami, 2009). β–galactosidase hydrolyses lactose to galactose and glucose, aiding lactose digestion in the intestine. When people have limited digestion or are lactose intolerant, the consumption of dairy products causes discomfort, gases and flatulence. Since LAB cultures can produce β–galactosidase, it is becoming important for the dairy industry to explore this property, in order to help lactose-intolerant consumers. Different *Lc. mesenteroides* strains showed β-galactosidase activity (Holt & Ricciardi, 2001; Rani & Agrawal, 2008; Monteagudo-Mera et al., 2011).

3.5 In vivo studies

The principal outcome of efficacy studies on probiotics should be consisting on proving benefits in human trials (FAO/WHO, 2002). The *in vivo* studies using *Lc. mesenteroides* as a potential probiotic strain is still scarce in comparison to the ones published about other *Lactobacillus* species, such as *Lactobacillus rhamnosus* or *Lactobacillus plantarum*. It is important to look carefully at the positive *in vivo* results, since most of them are inconsistent, unclear and controversial. However, improvements on the knowledge about the interactions of potential probiotic microorganisms in the GIT are important to understand better the behavior of these microorganisms in the host. Few *in vivo* studies about the potential probiotic *Lc. mesenteroides* strains have been described in the scientific literature.

Agarwal and Bhasin (2002) conducted a study in order to control the diarrhea in children (6 months to 5 years of age) by feeding them with fermented milk preparations (group 1: Actimel from Danone®, containing 10^8 CFU/g of each *Lactobacillus casei* DN-114001, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*; group 2: Indian Gahi, containing 10^8 CFU/g of each *Lactococcus lactis*, *Lactococcus lactis* subsp. *cremoris* and *Lc. mesenteroides* subsp. *cremoris*; and group 3: was given ultra-heat-treated yoghurt preparation - no live bacteria). Fermented milks that belong to group 1 and 2 showed a reduction in the duration of diarrhea in children in a community. However, the Indian Gahi presented a minor reduction, which the time taken to control diarrhea decreased by 0.3 day. In another study, the administration of symbiotic formula consisted of a combination of four probiotics (10^{11} CFU/g of each: *Pediococcus pentosaceus* 5, *Lc. mesenteroides* 32, *L. paracasei* subsp. *paracasei* 19, and *L. plantarum* 2), inulin, oat bran, pectin, and resistant starch as prebiotics was used during 15 treatment days of critically ill, mechanically ventilated, multiple trauma patients. The administration of this synbiotic formula in the patients exerted beneficial effects, decreasing the infection and sepsis rates. Besides, it improved the patient's immune response, who required ventilatory support for a shorter period of time and less intensive care treatment (Kotzampassi, Giamarellos-Bourboulis, Voudouris, Kazamias, & Eleftheriadis, 2006). Some clinical studies demonstrated that supplies of pre- and probiotics reduced the bacterial infection rates in patients

submitted to liver transplantation, antibiotic therapy following by pylorus-preserving pancreticoduodenectomy (PPPD), or patients who have underwent other high-risk surgeries (Rayes et al., 2005; Rayes et al., 2007; Rayes, Seehofera, & Seehofera, 2009).

The potential probiotic properties of *Lc. mesenteroides* cultures have also been studied in animal models. The effect of probiotic supplementation (10^7 CFU/g of each *Lc. mesenteroides* CLFP 196 and *Lactobacillus plantarum* CLFP 238) on the control of lactococcosis in rainbow trout for 30 days showed a significant reduction of fish mortality, from 78% in the control group to 46–54% in the probiotic groups (Vendrell et al., 2008). Furthermore, Pérez-Sánchez et al. (2011) conducted a study in order to understand the effect of some LAB (10^6 CFU/g of each *Lactobacillus plantarum* subsp. *plantarum* CLFP 3, *Lactococcus lactis* subsp. *cremoris* CLFP 25 *and Lc. mesenteroides* CLFP 68) on the control of lactococcosis for 36 days, as well as to assess the impact of probiotics on the expression of immune-related genes in the head kidney and intestine of rainbow trout (*Oncorhynchus mykiss*). The authors reported that only the diet containing *Lactobacillus plantarum* had significant lower fish mortality rates than diets containing *Lactococcus lactis*, *Lc. mesenteroides* or control. Moreover, *Lactobacillus plantarum* was able tostimulate the immune response of rainbow trout (Vendrell et al., 2008). Askarian, Kousha, Salma, and Ringø (2011) investigated the effect of *Lactobacillus curvatus* and *Lc. mesenteroides* (10^9 CFU/g in combination or alone), originally isolated from beluga (*Huso huso*) and Persian sturgeon (*Acipenser persicus*) GIT, respectively, on the growth, survival and digestive enzyme (amylase, lipase and protease) activities, and the population level of LAB in the GIT for 50 days. In Persian sturgeons study, highest specific growth rate, survival and improved intestinal enzyme activities were noted in the rearing group fed with *Lc. mesenteroides* (2×10^9 CFU/g). Similar results were found in beluga study, ie. *Lactobacillus curvatus* had positive effect. In another study, lactose-intolerance-induced rats were fed with diet supplemented with effective dose 10^8 CFU/mL of potential probiotic *Lc. mesenteroides* PLsr-$1_{(w)}$ strain, isolated from whey. After four days of probiotic feeding, the diarrhea disappeared. No changes in morphology and treatment related toxicity/bacterial translocation was observed on probiotic feeding (Rani, 2008). Besides, a reduction of acute

induced-peritonitis lung injury in rats had been shown with preoperative enteral administration of symbiotic composition (10^9 CFU/g of each *Pediococcus pentosaceus* 5, *Lc. mesenteroides* 32, *Lactobacillus paracasei* subsp. *paracasei* 19, *Lactobacillus plantarum* 2, inulin, oat bran, pectin and resistant starch as prebiotics), during 3 study weeks, using a cecal ligation and puncture model (Tok et al., 2007).

Further studies in animal models and humans, using alone *Lc. mesenteroides* strains should be done in order to confirm and comprise the behavior of this microorganism in the host GIT. Furthermore, the potential probiotic effects are always strain-dependent.

3.6 Therapeutic benefits studies of Lc. mesenteroides

The therapeutic benefits provided by potential probiotic LAB are frequently described in the literature. According to FAO/WHO (2002), a list of various tests (*in vitro* and *in vivo* trials) should be performed in order to assure that a strain is approved as a probiotic microorganim. Nonetheless, fulfill all of probiotic criteria is a hard task.

The study conducted by Kekkonen et al. (2008) confirmed a stimulation of the immune system by *Lc. mesenteroides* subsp. *cremoris* PIA2. The effect was more evident for the production of Th1 type cytokines (IL-12 and IFN-γ) than the one observed for some probiotic *Lactobacillus* strains (*Lactobacillus rhamnosus* GG/ATCC 53103, *Lactobacillus rhamnosus* Lc705/DSM 7061, *Lactobacillus helveticus* 1129, *Lactobacillus helveticus*).

The effect of the pre-treatment of LAB and bifidobacteria on inflammatory events, following by IL-8 induction by *Salmonella* Typhimurium DT104 was investigated by Carey and Kostrzynska (2012). *Lc. mesenteroides* IM080, *Bifidobacterium adolescentis* FRP 61, *Bifidobacterium longum* FRP 68 and FRP 69, *Bifidobacterium breve* FRP 334 and *Lactobacillus kefir* IM002 exerted an anti-inflammatory effect and led to a significant reduction in *Salmonella* Typhimurium DT104-induced IL-8 production by HT-29 cells. Balcázar et al. (2007) demonstrated a positive effect on humoral immune response following by oral probiotic administration (10^6 CFU/g of *Lc. mesenteroides*, *Lactococcus lactis* subsp. *lactis* and *Lactobacillus sakei*, isolated from salmonids) in brown trout (*Salmo trutta*). Further studies

about potential probiotic *Lc. mesenteroides* should be done in order to better comprise the therapeutic effects in animal models and humans; however, ethical constraints is one of the barriers that needs to be overtaken.

3.7 Antimicrobial activity against potentially pathogenic bacteria

The intestinal microbiota is a complex and dynamic ecossystem, which contains different bacterial species and representing an important contribution to the health of the host. The relationship between probiotic microorganisms and commensal intestinal bacteria remains uncertain (Larsen et al., 2011). The probiotic microbiota plays a key role by promoting the integrity of the epithelial barrier and the development of mucosal immunity (Correia, Liboredo, & Consoli, 2012). On the other hand, the mucosal surfaces of the GIT are particularly susceptible to the adherence and colonization of foodborne pathogens, such as *Salmonella* Typhymurium, *Listeria monocytogenes*, *Staphylococcus aureus*, *Shigella* spp. and *Escherichia coli* (Xu, Jeong, Lee, & Ahn, 2009). Potential probiotic *Lc. mesenteroides* strains (PLsr-$1_{(w)}$, DM1and PH1) produced antimicrobial activity against some pathogen species, such as *E coli* O157:H7 and other serotypes, *S. aureus*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Yersinia enterocolitica*, *Salmonella thyphi*, *Salmonella parathyphi* and *Shigella dysenteriae* (Rani & Agrawal, 2008; Ryu & Chang, 2013).

Probiotics can promote a positive balance in the GIT microbiota by increasing the concentration of beneficial microorganisms and inhibiting pathogenic bacteria (Correia, Liboredo, & Consoli, 2012). Different types of antimicrobial compounds can be produced by LAB, such as carbon dioxide, hydrogen peroxide, diacetyl, organic acids (lactic acid, acetic acid, and formic acid), ethanol, bacteriocins and others. These antimicrobial substances can inhibit undesirable microorganisms found in the GIT, and some of these antimicrobial compounds, such as bacteriocins, can also be used as biopreservatives by food industry. Moreover, the production of a physiologically restrictive environment (pH, redox potential, and hydrogen sulfide production) can inhibit the presence of undesirable microorganisms (Correia, Liboredo, & Consoli, 2012).

Nakamura et al. (2012) reported that competition to Caco-2 cells binding receptors and the antimicrobial activity against *Listeria monocytogenes* was observed for *Lc. mesenteroides* 1RM3, which presented strong inhibition against the pathogen invasion to Caco-2 cells. In addition, when A/J mice were orally infected with *Listeria monocytogenes*, the recovery of the pathogen from the spleen was suppressed by the administration of water containing 9 log CFU/mL of *Lc. mesenteroides* - 1RM3 cells. The inhibitory effects were also shown by heat-killed of 1RM3 cells. An *in vitro* cell lines study showed an antiviral activity of *Lc. mesenteroides* YML003 against low-pathogenic avian influenza (H9N2), as well as for *in vivo* specific pathogen-free (SPF) chickens (Seo et al., 2012).

Another *in vitro* study, using pure cultures of *Lc. mesenteroides* MTCC 5442 and *Bacillus subtilis* natto RG4365, showed that each probiotic strain was able to inhibit the planktonic growth of *Vibrio cholerae*, as well as its ability to form biofilms and adhere to extracellular protein matrix (fibronectin, Fn), which may function *in vivo* as initial gateway for the pathogen. When both cultures were used together in the *ragi* (cereal) malt, the antimicrobial activity against *V. cholerae* was enhanced due to a synergistic effect (Vidyalaxme, Rovetto, Grau, & Agrawal, 2012).

3.8 Behavior of Lc. mesenteroides in food as a probiotic microorganism

The presence of LAB plays an important role in food preservation, health benefits and production of antimicrobial compounds. Probiotic LAB are commonly applied in dairy products, being widely used in industrial applications, mainly as starter or complementary cultures. The probiotic candidate strain should survive to food processing and biological stresses, which includes extreme temperatures and pH values, as well as osmotic and oxidative stresses, and bacteriophage attack (Giraffa, 2012). Moreover, depending on the product, other requirements should be fulfilled, like production of aroma, acidification and viability during shelf-life.

Leuconostoc spp. can form significant amounts of diacetyl from citrate in milk, which is an important compound produced mainly by *Lc. mesenteroides* subsp. *cremoris* and related to

aroma and flavor. This substance plays an important role in the production of butter and cheese. The production of exopolysaccharides by this specie is ordinary. Its slime-forming property is commonly used by dairy industry, especially in the production of thick viscous fermented milk products. Furthermore, this specie can grow over a wide range of temperatures and tolerate higher salt concentrations up to 7% NaCl (Hemme & Foucaud-Scheunemann, 2004). Despite these technological characteristics, studies about *Lc. mesenteroides* as a potential probiotic microorganism are rare in the literature. Probiotic strains *Lactobacillus plantarum* L4 and *Lc. mesenteroides* LMG 7954 were applied for fermentation control of cabbage heads. The starter cultures applied for cabbage heads fermentation allowed a decrease of NaCl concentrations from 4.0% to 2.5% (w/v), and acceleration of fermentation process by 14 days. The produced sauerkraut heads were considered as a probiotic, as viable probiotic cultures cells counts in the final product were higher than 10^6 CFU/g (Beganović et al., 2011).

4. Studies about bacteriocinogenic *Lc. mesenteroides*

4.1 Bacteriocins produced by Lc. mesenteroides

The bacteriocins produced by *Lc. mesenteroides* can be applied as biopreservatives in food industry; however, the process that goes from discovery of a new bacteriocins until its final application in food systems remains a challenge. Most of bacteriocins produced by *Lc. mesenteroides* shows anti-*Listeria* activity (Daba et al., 1991; Héchard, Derjard, Letellier, & Cenatiempo, 1992; Hastings, Stiles, & Von Holy, 1994; Revol-Junelles et al., 1996; Papathanasopoulos et al., 1997; Guyonnet, Fremaux, Cenatiempo, & Berjeaud, 2000; Todorov & Dicks, 2004; Aymerich et al., 2005; Vélez et al., 2007; Trias, Badosa, Montesinos, & Bañeras, 2008; Xiraphi et al., 2008; Ratti, Gomes, Martinez, Souza, & De Martinis, 2010; Osmanagaoglu & Kiran, 2011; Sip, Więckowicz, Olejnik-Schmidt, & Grajek, 2012; Wulijideligen et al., 2012). *Listeria monocytogenes* is a public health concern since this microorganism is a foodborne pathogen that survives under different environment conditions and can cause abortions, gastrointestinal diseases, septicaemia and exhibits high mortality rates

in, children, eldery, immunocompromised and transplanted organ individuals (Sip, Więckowicz, Olejnik-Schmidt, & Grajek, 2012). In addition, some unusual bacteriocins produced by *Lc. mesenteroides* showed antimicrobial activity against Gram-negative bacteria such as *Salmonella* Thyphimurium and *E. coli* (Vélez et al., 2007; Masuda et al., 2011), Gram-positive such as bacteria *Bacillus cereus* (Masuda et al., 2011) and *S. aureus* (Todorov & Dicks, 2004; Trias, Badosa, Montesinos, & Bañeras, 2008).

The complex biochemical nature of the bacteriocins, its stability and interactions with food matrices, the best way to obtain high amounts of these compounds and how to diminish the losses in the purification processes are just some of the aspects that should be observed in order to enable bacteriocins application as food biopreservatives.

Different species of *Lc. mesenteroides*, isolated from various matrices and environments were able to produce the same bacteriocins: mesentericin B105 and mesentericin Y105, leucocin A- B- C-TA33a and leucocin A-UAL 187 (Hastings, Stiles, & Von Holy, 1994; Papathanasopoulos et al., 1997; Héchard, Derjard, Letellier, & Cenatiempo, 1992; Xiraphi et al., 2008; Abriouel, Martín-Platero, Maqueda, Valdivia, & Martínez-Bueno, 2008; Trias, Badosa, Montesinos, & Bañeras, 2008). It is also important to point out that some encoding genes for bacteriocins seem to be very well conserved. Molecular studies indicated that the genes encoding mesentericins Y105 and B105 are located on the chromosome, which leads to a higher stability of the genes, with lower chances of occurrence of mutation or losses (Osmanagaoglu & Kiran, 2011).

However, the production of different bacteriocins were observed for *Lc. mesenteroides* strains: mesenterocin 5 (Daba et al., 1991); leucocin A-UAL 187 (Hastings, Stiles, & Von Holy, 1994); mesenterocin 52A and 52B (Revol-Junelles et al., 1996); leucocin A- B- C-TA33a (Papathanasopoulos et al., 1997); leucocin C (Fimland, Sletten, & Nissen-Meyer, 2002); mesentericin ST99 (Todorov & Dicks, 2004); bacteriocin ST33LD (Todorov & Dicks, 2005); mesenterocin E131 (Xiraphi et al., 2008); and leucocyclicin Q (Masuda et al., 2011).

4.2 Bacteriocins produced by Lc. mesenteroides in food

Bacteriocins produced by LAB can be applied in food by three different ways, introduction of producer strains, addition of partial purified or purified concentrates, and utilization of fermented ingredients (Cotter, Hill, & Ross, 2005; Balciunas et al., 2013). However, the production of bacteriocins can be affected by different factors, such as NaCl concentration, temperature, enzymes, pH, food matrices and food contamination.

Various studies have described bacteriocins produced by *Lc. mesenteroides.* In addition, few reports described their potential for application in food biopreservation. Metaxopoulos, Mataragas and Drosinos (2002) inoculated sliced cooked cured pork shoulder with the bacteriocin-producing strains *Lc. mesenteroides* L124 and *L. curvatus* L422. In the non-inoculated samples, under vacuum packaging, the growth of spoilage microflora was observed, while in the inoculated ones the counts of *Brochothrix thermosphacta* and enterococci reduced (1.5 log CFU/g) during storage.

Studies have shown that bacteriocins produced by *Lc. mesenteroides* can be used in different types of food application. For example, the excess of malolactic fermentation (production of L-malic acid from L-lactic acid) by LAB can yield undesirable flavor compounds in wine fermentation. The bacteriocin-like inhibitory substance produced by *Lc. mesenteroides* subsp. *cremoris* displayed antimicrobial activity against malolactic activity of autochthonous LAB in wines (Yurdugül & Bozoglu, 2002). *Lc. mesenteroides* strains presented potential application for inhibition of *L. monocytogenes* in wounds of Golden Delicious apples and Iceberg lettuce leaf cuts. The use of these LAB strains, as bioprotective agents in food matrices, provided inhibitory results against the pathogenic microorganism. This application is supported by the safe nature of the mentioned bacterial group. *Lc. mesenteroides* produced Class II bacteriocins with high anti-listerial activity, identical to mesentericin Y105. Moreover, the application of these strains in fresh fruits and vegetables did not cause sensory modifications although longer assays should be performed to confirm that the application of these bioprotective strains do not alter the shelf-life of the product (Trias, Badosa, Montesinos, & Bañeras, 2008).

An interesting field that is still in progress is the use of bioengineering techniques, which can create a new era of powerful hybrid bacteriocins with a wide anti-bacterial spectrum and high specific activity (Acuña, Morero, & Bellomio, 2011). A fusion of different bacteriocin-encoding genes with different targets can create a new bacteriocin that could act more efficiently in food biopreservation. A novel recombinant hybrid peptide, combining enterocin CRL35 and microcin V (named Ent35–MccV), displayed an antimicrobial activity against enterohemorrhagic *E. coli* and *L. monocytogenes* clinical isolates (Acuña, Picariello, Sesma, Morero, & Bellomio, 2012). More details about the development of wide-spectrum hybrid bacteriocins were reported by Acuña, Morero and Bellomio (2011).

It is noteworthy to highlight that, once the bacteriocins are useful as biopreservatives in food industry, the potential of target species for the development resistance to these compounds is another subject to concern. It is important to emphasize that bacteriocins should be an additional tool used in food systems to protect against undesirable microorganisms and must be applied in combination with other barriers, such as pH control, a_w, temperature and oxygen, in the hurdle technology (Cotter, Hill, & Ross, 2005). To understand better the hurdle technology and its use in combination with antimicrobial compounds, Leistner and Gorris (1995) and Leistner (2000) published two reviews about the subject. In addition, it is important to stress out that the use of the hurdle techniques does not replace the systematic preventive approach for food safety, such as the Hazard Analysis and Critical Control Points (HACCP) and the Good Manufacturing Practice (GMP).

5. The other side of *Lc. mesenteroides*

5.1 Pathogenic strains

Few reports on systemic infections caused by pathogenic *Lc. mesenteroides* are described in the literature. The characterization of microbiota in coeliac disease in children was studied by Sanz et al. (2007). The diversity of the faecal microbiota was significantly higher in coeliac patients than in healthy controls. The prevalence of the species *Lactobacillus curvatus*,

Lc. mesenteroides and *Lc. carnosum* was higher in coeliac patients than in healthy controls. Opposite results were found for *Lactobacillus casei* group, which was more prevalent in healthy controls. Most LAB species detected in coeliac patients were probably transient (allochthonous) while in healthy-samples species of the *Lactobacillus casei* group could be regarded as both endogenous and food-related bacteria (Sanz et al., 2007). However, at present, little is known about the potential role of the microbiota in coeliac patients.

Isolate cases of *Lc. mesenteroides* as emerging pathogen were reported. Some authors commented that *Lc. mesenteroides* strains were related with the occurrence of bacteremia (Lee et al., 2011), nosocomial infection (Bou et al., 2008), short bowel syndrome (Florescu, Hill, Sudan, & Iwen, 2008), brain abscess (Albanese et al., 2006), short GIT syndrome, parenteral nutrition and continuous enteral feeding (Jofré et al., 2006), endocarditis (Vásquez et al., 1998), septicemia (Kikuchi et al., 1994), meningitis (Friedland, Snipelisky, & Khoosal, 1990) and odontogenic infection (Wenocur, Smith, Vellozzi, Shapiro, & Isenberg, 1988).

The gateway for *Leuconostoc*-caused infections is still unclear; however, several authors have suggested the skin, catheter, intra-abdominal origin, GIT, combined blender (used to prepare infant formula) and enteral feeding as possible pathways.

In spite of that, little is known about the pathogenic *Lc. mesenteroides* effects in patients, and most of clinical symptoms are fever, leukocytosis and gastrointestinal disorders. Because of the rarity of infections, we have insignificant knowledge about the harmful effects or optimal therapy for these condions (Florescu, Hill, Sudan, & Iwen, 2008). The great majority cases of pathogenic *Lc. mesenteroides* are related to underlying diseases and immunocompromised patients. Moreover, the intake of dairy products containing this microorganism is not related to any disease (Lee et al., 2011).

5.2 Antibiotic resistance and virulence genes

Transferable resistance genes may pose a risk for the utilization of LAB in food processing, as they can be transferred from pathogenic bacteria to non-pathogenic organisms (Aymerich et al., 2005; Devirgiliis, Barile, & Perozzi, 2011). LAB in food may act as reservoirs

of antibiotic resistance genes (Ammor & Mayo, 2007). These genes may be transferred by different bacteria from normal GIT microbiota, normal GIT microbiota and pathogens, or between pathogens, which use this strategy as a way of adapting to an ever-changing environment (Van Rennen & Dicks, 2011). It is noteworthy to highlight that, in some cases, the resistance to specific antibiotics can be desirable, like in treatments involving the antibiotic-induced diarrhea (Argyri et al., 2013).

Similar to *Lactobacillus* and *Pediococcus* genera, *Leuconostoc* spp. has an intrinsic resistance to vancomycin (Divya, Varsha, & Nampoothiri, 2012; Muñoz-Atienza et al., 2013), due to particular characteristics of its cell wall that presents D-lactate instead of a D-alanine in the peptidoglycan layer (Hemme & Foucaud-Scheunemann, 2004). In addition, the intrinsic or natural resistance is encoded by chromosomal genes, therefore, non-transferable (Argyri et al., 2013). Previous studies with *Lc. mesenteroides*, isolated from different sources, reported the presence of vancomycin-resistance among the tested strains (Aymeric et al., 2006; Vay et al., 2007; Aswathy, Ismail, John, & Nampoothiri, 2008; Rojo-Bezares et al., 2008; Cardamone et al., 2011; Pérez-Sánchez et al., 2011; Shobharani & Agrawal, 2011; Argyri et al., 2013; Ryu & Chang, 2013).

Natural (horizontal) gene transference may also happen (Dicks, Todorov, & Franco, 2009), but it has been rarely found (Hemme & Foucaud-Scheunemann, 2004). Interesting reviews about antibiotic resistance in LAB and horizontal gene transfer were published by Dicks, Todorov, and Franco (2009) and Van Rennen and Dicks (2011). The literature describes several cases of *Lc. mesenteroides* resistance to antibiotics, other than vancomycin (Table 1). In contrast, a study conducted by Vermeiren, Devlieghere, and Debevere (2006), showed that strain *Lc. mesenteroides* LM4 did not show resistance to any of the 11 antibiotics tested.

Genes encoding virulence factors commonly found in *Enterococcus* genus, such as cytolysin (*cylA* and *cylB*), aggregation substance (*asp1*, *asc10* and *asa1*), *Enterococcus* surface protein (*esp*), adhesins (*ace* and *acm*), adhesin-like endocarditis antigens (*efaA*), gelatinase (*gelE*), hyaluronidase (*hyl*) and biogenic amines are not commonly described for *Lc.*

mesenteroides strains. *Lc. mesenteroides* 875 did not present gelatinase and hyaluronidase activity (Monteagudo-Mera et al., 2011).

Table 1 Profiles of antibiotics for resistance for *Lc. mesenteroides* strains isolated from different sources.

Food matrix	Resistance	References
Spanish goats' milk cheese	ampicillin, augmentin, cefoxitin, cephalotoxin, oxacillin, vancomycin, teicoplanin, cloranphenicol, clindamycin, rifampicin and tetracycline	Herreros et al., 2005
Fermented sausage	gentamicin, ampicillin, penicillin G and tetracycline	Aymeric et al., 2006
Clinical isolates	trimethoprim–sulfamethoxazole	Vay et al., 2007
Olive brine	nalidixic acid, sulpfamethoxazole, neomycin, tobramycin, ciprofloxacin, amikacin, streptomycin, metronidazole and sulphafurazole	Todorov & Dicks, 2008
Whey	cephalotin, gentamycin and oxacillin	Rani & Agrawal, 2008
Intestine of rainbow trout	ampicillin, clindamycin, enrofloxacin, flumequine, kanamycin, nalidixic acid, oxolinic acid, penicillin, tetracycline and trimethoprim/sulfamethoxazole	Pérez-Sánchez et al., 2011
Cheddar cheese	tetracycline, gentamicin, neomycin, streptomycin and sulfisoxazole	Shobharani & Agrawal, 2011
Intestinal microflora of snakehead fish	Streptomycin	Allameh, Daud, Yusoff, Saad, & Ideris, 2012
Fermented olives	Kanamycin	Argyri et al., 2013

Biogenic amines are generated by decarboxylation of the corresponding amino acids through substrate-specific enzymes derived from microorganisms present in food. Cadaverine and putrescine can contribute to food spoilage in several products, and they have toxicological effects, observed in many critical functions for both human and animals by the consumption of foods containing high amounts of these compounds (De Las Rivas, Marcobal, & Muñoz, 2005). In a study conducted by Pircher, Bauer, and Paulsen (2007), isolated strains of *Lc.*

mesenteroides were unable to produce more than 10 mg/l of amine at high inoculum level, in a medium rich in nutrients, and under *in vitro* conditions. In another study with *Leuconostoc* spp. strains, just 1/76 strains of *Lc. mesenteroides* subsp. *mesenteroides* produced >100 mg/l of putrescine and cadaverine. The toxic levels seemed to depend on the characteristics of different individuals (Linares, Martín, Ladero, Alvarez, & Fernández, 2011). In contrast, *Lc. mesenteroides* strains isolated from different foods, such as fermented sausage (Aymerich et al., 2005), processed fish from Eastern Himalayas (Thapa, Pal, & Tamang, 2006), Spanish cheese (Nieto-Arribas, Seseña, Poveda, Palop, & Cabezas, 2010), meat products from Eastern Himalayas (Rai, Tamang, & Palni, 2010), musts (Mesas, Rodríguez, & Alegre, 2011) and traditional, starter-free cheeses (Alegría, Delgado, Flórez, & Mayo, 2013) did not produce biogenic amines.

Linares, Martín, Ladero, Alvarez, and Fernández (2011) published a review regarding relevant aspects of biogenic amines production in dairy products, such as the effect of environmental conditions, the microorganisms that produce them, the genetic organization and regulation of the biosynthetic pathways involved, and the available methods for detecting the presence of either these compounds or the biogenic-amines-producing microorganisms.

5.3 Hemolytic potential

The presence of certain enzymes in potential probiotic microorganisms has been related to pathogenicity for human beings. For this reason, the lack/low levels of such enzymes activity is a positive aspect for strains that are intended to be used as probiotics or starters cultures (Monteagudo-Mera et al., 2011). Hemolytic potential is not desirable in probiotic cultures. Some studies comment that strains of *Lc. mesenteroides* presents non-hemolytic characteristic when tested on sheep blood agar (Florescu, Hill, Sudan, & Iwen, 2008), which was also reported by *Lc. mesenteroides* DM1 and *Lc. mesenteroides* PH1 isolated from *kimchi* (Ryu & Chang, 2013) and *Lc. mesenteroides* 875 isolated from raw ewe's milk cheese (Monteagudo-Mera et al., 2011).

The absence of β-glucosidase, N-acetyl-β-glucosaminidase, and β-glucuronidase enzymes is one of the selection criteria for probiotic LAB to be used in food industry. The activity of these enzymes can be harmful and are associated with health disorders or intestinal diseases, such as the ability to convert procarcinogens to carcinogens in the colon (Giraffa, 2012; Saarela, Mogensen, Fondén, Mättö, & Mattila-Sandholm, 2000). Studies with *Lc. mesenteroides* strains isolated from different sources have shown the absence of β-glucosidase, N-acetyl-β-glucosaminidase and β-glucuronidase enzymes (Lei, Amoa-Awua, & Brimer, 1999; Mesas, Rodríguez, & Alegre, 2011; Monteagudo-Mera et al., 2011; Ryu & Chang, 2013). However, some *Lc. mesenteroides* strains isolated from fermented cassava (Obilie, Tano-Debrah, & Amoa-Awua, 2004; Kostinek et al., 2007), sourdoughs (Zotta, Ricciardi, & Parente, 2007), meat products from Eastern Himalayas (Rai, Tamang, & Palni, 2010), musts (Mesas, Rodríguez, & Alegre, 2011), dairy samples (Monteagudo-Mera et al., 2011) and *kimchi* (Ryu & Chang, 2013) showed positive β-glucosidase activity.

5.4 Spoilage strains

Lc. mesenteroides can be considered as a spoilage agent of certain foods, such as meat products, alcoholic beverages, sugar cane and beet, causing a critical impact on their quality (Stiles & Holzapfel, 1997). The main origin of contamination of processed producers with *Lc. mesenteroides* is the environment (plant surface and soil), but natural contamination of the raw material can also occur.

The presence of this microorganism in wine, cachaça and sugar cane can result in the development of undesirable characteristics, such as the formation of exopolysaccharide slimes, producing ropy products (dextrans and other polysaccharides), and the development of off-flavor (acetic acid, diacetyl and mannitol) (Hutkins, 2006; Petri, Pfannebecker, Fröhlich, & König, 2013).

In the sugar production, the first step is the removal of the leafy parts from the sugar canes. After this, the raw material is ferried to the mills. During these steps, there is a considerable contamination with *Lc. mesenteroides*, which leads to the production of a gum that

causes problems during extraction of sugar cane (Bamforth, 2005). When beet is used as raw material to sugar extraction, the presence of *Lc. mesenteroides* subsp. *dextranicum* is the major problem. This microorganism is able to produced extracellular slimes, which can cause filtration problems in sugar factories (Strausbaugh & Gillen, 2008).

Concerning alcoholic fermentation, *Leuconostoc* strains are frequently associated with problems during the process, such as acid production and yeast flocculation. This phenomenon can cause industrial problems, such as interruption of cell centrifugation, occurrence of phase separation between yeast cells and substrate, and yeast loss at the bottom of the reactor, mixed with other solid particles (Oliva-Neto & Yokoya, 1998). Nonetheless, *Leuconostoc* genus is sensitive to ethanol and presents a short-life inside the tanks (Meneghin, Reis, Almeida, & Ceccato-Antonini, 2008).

In wine fermentation, the glycerol oxidation carried out by *Lc. mesenteroides* and lactobacilli strains generates acrolein. This aldehyde reacts with tannins and anthocyanins, forming bitter compounds (Hutkins, 2006). Montersino, Prieto, Muñoz, and De Las Rivas (2008) reported that *Lc. mesenteroides* strains, isolated from grape juice or wine, were able to produce exopolysaccharides. These compounds are responsible for an alteration known as ropiness or oiliness, characterized by the appearance of viscous thick and oily texture. Although these defects do not provoke significative changes in taste, it makes the unpleasant product to the palate.

Other different studies have confirmed the spoilage effect assigned by *Lc. mesenteroides*. The growth of this microorganism in processed cactus pear fruit was not affected in modified atmospheres during the storage. However, the spoilage effect was not described in the study of Corbo et al. (2004).

Some studies demonstrated that high-pressure treatment and pasteurization were able to delay the occurrence of spoilage caused by *Weissella viridescens* and *Lc. mesenteroides* in blood sausages. Besides, the authors commented that *Lc. mesenteroides* produced sour smell, and milky exudates that were not as viscous as slime and cause the appearance of green spots (Diez, Jaime, & Rovira, 2009; Diez, Björkroth, Jaime, & Rovira, 2009). The presence of *Lc.*

mesenteroides in meat packaged under modified atmosphere can cause the increase of carbon dioxide levels, especially in refrigerated raw meat, which is a characteristic of heterofermentative LAB. The presence of *Lc. mesenteroides* B242 in modified atmosphere packaged, minced meat, with and without oregano essential oil, was reported by Doulgeraki, Paramithiotis, Kagkli, and Nychas (2010). However, this strain did not provoke any undesired effect in the product. On the other hand, the presence of *Lc. mesenteroides* in air stored (4 and 10 ^{o}C) Greek taverna sausage caused the appearance of slime and gas production in the product (Samelis & Georgiadou, 2000).

Although *Lc. mesenteroides* spoilage is confirmed in different food matrices, further studies should be carried out in order to better understand the behavior of this microorganism under the mentioned conditions. Nonetheless, it is important to point out the relevance of the use of hurdle techniques and improvement of the hygienic conditions, which can help to avoid the presence of spoilage *Lc. mesenteroides* in these matrices.

6. Conclusion

Lc. mesenteroides presents promisor characteristics, such as probiotic potential and bacteriocin production. Nonetheless, rare strains exhibit pathogenic profile and cause spoilage in different food matrices. The two faces of *Lc. mesenteroides* show that further studies should be performed to better understand the behavior of this microorganism in different fields, and the judgment of every bacterial species needs to be evaluated based on each specific strain.

Acknowledgments

The authors are grateful to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Brazil, Project N° 2010/09302-1), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil) for the financial support.

References

Abd El-Salam, M. H., El-Shafei, K., Sharaf, O. M., Effat, B. A., Asem, F. M., & El-Aasar, M. (2010). Screening of some potentially probiotic lactic acid bacteria for their ability to synthesis conjugated linoleic acid. *International Journal of Dairy Technology, 63,* 62-69.

Abriouel, H., Martín-Platero, A., Maqueda, M., Valdivia, E., & Martínez-Bueno, M. (2008). Biodiversity of the microbial community in a Spanish farmhouse cheese as revealed by culture-dependent and culture-independent methods. *International Journal of Food Microbiology, 127*, 200–208.

Acuña, L., Morero, R. D., & Bellomio, A. (2011). Development of wide-spectrum hybrid bacteriocins for food biopreservation. *Food Bioprocess Technology, 4*, 1029–1049.

Acuña, L., Picariello, G., Sesma, F., Morero, R. D., & Bellomio, A. (2012). A new hybrid bacteriocin, Ent35–MccV, displays antimicrobial activity against pathogenic Gram-positive and Gram-negative bacteria. *FEBS Open Bio, 2*, 12–19.

Adams, C. (2010). The probiotic paradox: live and dead cells are biological response modifiers. *Nutrition Research Reviews, 23*, 37–46.

Agarwal, K. N., & Bhasin, S. K. (2002). Feasibility studies to control acute diarrhea in children by feeding fermented milk preparations Actimel and Indian Dahi. *European Journal of Clinical Nutrition, 56*, S56-S59.

Albanese, A., Spanu, T., Sali, M., Novegno, F., D'inzeo, T., Santangelo, R., et al. (2006). Molecular identification of *Leuconostoc mesenteroides* as a cause of brain abscess in an immunocompromised patient. *Journal of Clinical Microbiology, 44*, 3044-3045.

Albesharat, R., Ehrmann, M. A., Korakli, M., Yazaji, S., & Vogel, R. F. (2011). Phenotypic and genotypic analyses of lactic acid bacteria in local fermented food, breast milk and faeces of mothers and their babies. *Systematic and Applied Microbiology, 34*, 148–155.

Alegría, A., Delgado, S., Flórez, A. B., & Mayo, B. (2013). Identification, typing, and functional characterization of *Leuconostoc* spp. strains from traditional, starter-free cheeses. *Dairy Science and Technology, 93*, 657–673.

Allameh, S. K., Daud, H., Yusoff, F. M, Saad, C. R., & Ideris, A. (2012). Isolation, identification and characterization of *Leuconostoc mesenteroides* as a new probiotic from intestine of snakehead fish (*Channa striatus*). *African Journal of Biotechnology, 11*, 3810-3816.

Amara, A. A., & Shibl, A. (2013). Role of probiotics in health improvement, infection control and disease treatment and management. *Saudi Pharmaceutical Journal*, in press.

Ammor, S. M., & Mayo, B. (2007). Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: an update. *Meat Science, 76*, 138-146.

Argyri, A. A., Zoumpopoulou, G., Karatzas, K. A. G., Tsakalidou, E., Nychas, G. J. E., Panagou, E. Z. et al. (2013). Selection of potential probiotic lactic acid bacteria from fermented olives by *in vitro* tests. *Food Microbiology, 33*, 282-291.

Askarian, F., Kousha, A., Salma, W., & Ringø, E. (2011). The effect of lactic acid bacteria administration on growth, digestive enzyme activity and gut microbiota in Persian sturgeon (*Acipenser persicus*) and beluga (*Huso huso*) fry. *Aquaculture Nutrition, 17*, 488-497.

Aswathy, R. G., Ismail, B., John, R. P., & Nampoothiri, K. M. (2008). Evaluation of the probiotic characteristics of newly isolated lactic acid bacteria. *Applied Biochemistry and Biotechnology, 151*, 244–255.

Aymerich, T., Martín, B., Garriga, M., Vidal-Carou, M. C., Bover-Cid, S., & Hugas, M. (2005). Safety properties and molecular strain typing of lactic acid bacteria from slightly fermented sausages. *Journal of Applied Microbiology, 100*, 40–49.

Balcázar, J. L., De Blas, I., Ruiz-Zarzuela, I., Vendrell, D., Calvo, A. C., Márquez, I., et al. (2007). Changes in intestinal microbiota and humoral immune response following probiotic administration in brown trout (*Salmo trutta*). *British Journal of Nutrition, v. 97*, 3, 522-527.

Balciunas, E. M., Martinez, F. A. C., Todorov, S. D., Franco, B. D. G. M., Converti, A., & Oliveira, R. P. S. (2013). Novel biotechnological applications of bacteriocins: a review. *Food Control, 32*, 134-142.

Bamforth, C. W. (2005). Distilled Alcoholic Beverages. In C. W. Bamforth (Eds.), *Food, fermentation and micro-organisms* (pp. 122-132). Iowa: Blackwell Science Ltd.

Beganović, J., Pavunc, A. L., Gjuračić, K., Špoljarec, M., Šušković, J., & Kos, B. (2011). Improved sauerkraut production with probiotic strain *Lactobacillus plantarum* L4 and *Leuconostoc mesenteroides* LMG 7954. *Journal of Food Science, 76*, M124-M129.

Begley, M., Hill, C., & Gahan, C. G. M. (2006). Bile salt hydrolase activity in probiotics. *Applied and Environmental Microbiology, 72*, 1729-1738.

Bendimerad, N., Kihal, M., & Berthier, F. (2012). Isolation, identification, and technological characterization of wild leuconostocs and lactococci for traditional Raib type milk fermentation. *Dairy Science and Technology, 92*, 249-264.

Bou, G., Saleta, J. L., Nieto. J. A. S., Tomás, M., Valdezate, S., Sousa, D., et al. (2008). Nosocomial outbreaks caused by *Leuconostoc mesenteroides* subsp. *mesenteroides*. *Emerging Infection Diseases, 14*, 968-971.

Cardamone, L., Quiberoni, A., Mercanti, D. J., Fornasari, M. E., Reinheimer, J. A., & Guglielmotti, D. M. (2011). Adventitious dairy *Leuconostoc* strains with interesting technological and biological properties useful for adjunct starters. *Dairy Science and Technology, 91*, 457-470.

Carey, C. M., & Kostrzynska, M. (2012). Lactic acid bacteria and bifidobacteria attenuate the proinflammatory response in intestinal epithelial cells induced by *Salmonella enterica* serovar Typhimurium. *Canadian Journal of Microbiology, 59*, 9-17.

Chang, J. H., Shim, Y. Y., Cha, S. –K, & Chee, K. M. (2010). Probiotic characteristics of lactic acid bacteria isolated from *kimchi*. *Journal of Applied Microbiology, 109*, 220–230.

Cho, G. S., & Do, H. K. (2006). Isolation and identification of lactic acid bacteria isolated from a traditional jeotgal product in Korea. *Ocean Science Journal, 41*, 113-119.

Collado, M. C., Surono, I., Meriluoto, J., & Salminen, S. (2007). Indigenous dadih lactic acid bacteria: cell-surface properties and interactions with pathogens. *Journal of Food Science, 72*, M89-M93.

Corbo, M. R., Altieri, C., D'amato, D., Campaniello, D., Del Nobile, M. A., & Sinigaglia, M. (2004). Effect of temperature on shelf life and microbial population of lightly processed cactus pear fruit. *Postharvest Biology and Technology, 31*, 93-104.

Correia, M. I. T. D., Liboredo, J. C., & Consoli, M. L. D. (2012). The role of probiotics in gastrointestinal surgery. *Nutrition, 28*, 230-234.

Cotter, P. D., Hill, C., & Ross, R. P. (2005). Bacteriocins: developing innate immunity for food. *Nature Reviews Microbiology, 3*, 777-788.

Daba, H., Pandian, S., Gosselin, J. F., Simard, R. E., Huang, J., & Lacroix, C., (1991). Detection and activity of a bacteriocin produced by *Leuconostoc mesenteroides*. *Applied and Environmental Microbiology, 57*, 3450-3455.

De Azevedo, M. S. P., Innocentin, S., Dorella, F. A., Rocha, C. S., Mariat, D., Pontes, D. S., et al. (2013). Immunotherapy of allergic diseases using probiotics or recombinant probiotics. *Journal of Applied Microbiology, 115*, 319-333.

De Las Rivas, B., Marcobal, A., & Muñoz, R. (2005). Improved multiplex-PCR method for the simultaneous detection of food bacteria producing biogenic amines. *FEMS Microbiology Letters, 244*, 367-372.

Deepika, G., Rastall, R. A., & Charalampopoulos, D. (2011). Effect of food models and low-temperature storage on the adhesion of *Lactobacillus rhamnosus* GG to Caco-2 cells. *Journal of Agricultural and Food Chemistry, 59*, 8661-8666.

Devirgiliis, C., Barile, S., & Perozzi, G. (2011). Antibiotic resistance determinants in the interplay between food and gut microbiota. *Genes and Nutrition, 6*, 275-284.

Dicks, L. M. T., Todorov, S. D., & Franco B. D. G. M. (2009). Current status of antibiotic resistance in lactic acid bacteria. In A. R. Bonilla, & K. P. Muniz (Eds.), *Antibiotic resistance: causes and risk factors, mechanisms and alternatives. Pharmacology—research, safety testing and regulation* (pp. 379–425). New York: Nova Publisher.

Diez, A. M., Björkroth, J., Jaime, I., & Rovira, J. (2009). Microbial, sensory and volatile changes during the anaerobic cold storage of *morcilla de Burgos* previously inoculated with *Weissella viridescens* and *Leuconostoc mesenteroides. International Journal of Food Microbiology, 131*, 168–177.

Diez, A. M., Jaime, I., & Rovira, J. (2009). The influence of different preservation methods on spoilage bacteria populations inoculated in *morcilla de Burgos* during anaerobic cold storage. *International Journal of Food Microbiology, 132,* 91-99.

Divya, J. B., Varsha, K. K, & Nampoothiri, K. M. (2012). Newly isolated lactic acid bacteria with probiotic features for potential application in food industry. *Applied Biochemistry and Biotechnology, 167*, 1-11.

Dobson, A., Cotter, P. D., Ross, R. R., & Hill, C. (2012). Bacteriocin production: a probiotic trait? *Applied and Environmental Microbiology, 78*, 1-7.

Doulgeraki, A. I., Paramithiotis, S., Kagkli, D. M., & Nychas, G. J. E. (2010). Lactic acid bacteria population dynamics during minced beef storage under aerobic or modified atmosphere packaging conditions. *Food Microbiology, 27*, 1028-1034.

European Economic Community (1983). European Economic Community Commission Directive. *Official Journal of the European Union 255*, 1–6, 83/463/EEC.

FAO/WHO (2002) Guidelines for the evaluation of probiotics in food. Report of a joint Food and Agriculture Organization (FAO) of the United Nations/World Health Organization (WHO) working group on drafting guidelines for the evaluation for the probiotics in food. Available at: http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf

Fimland, G., Sletten, K., & Nissen-Meyer, J. (2002). The complete amino acid sequence of the pediocin-like antimicrobial peptide leucocin C. *Biochemical Biophysical Research Communications, 295*, 826–827.

Florescu, D., Hill, L., Sudan, D., & Iwen, P. C. (2008). *Leuconostoc* bacteremia in pediatric patients with short bowel syndrome. *Pediatric Infectious Disease Journal, 27*, 1013-1019.

Fontana, L., Bermudez-Brito, M., Plaza-Diaz, J., Muñoz-Quezada, S., & Gil, A. (2013). Sources, isolation, characterization and evaluation of probiotics. *British Journal of Nutrition, 109*, S35-50.

Friedland, I. R., Snipelisky M., & Khoosal M. (1990). Meningitis in a neonate caused by *Leuconostoc* sp. *Journal of Clinical Microbiology, 28*, 2125–2126.

Giraffa, G. (2012). Selection and design of lactic acid bacteria probiotic cultures. *Enginnering Life Science, 12*, 391–398.

Guyonnet, D., Fremaux, C., Cenatiempo, Y., & Berjeaud, J. M. (2000). Method for rapid purification of class IIa bacteriocins and comparison of their activities. *Applied and Environmental Microbiology, 66*, 1744-1748.

Hastings, J. W., Stiles, M. E., & Von Holy, A. (1994). Bacteriocins of *Leuconostocs* isolated from meat. *Food Microbiology, 24*, 75-81.

Héchard, Y., Derjard, B., Letellier, F., & Cenatiempo, Y. (1992). Characterization of mesentericin Y105, an anti-Listeria bacteriocin from *Leuconostoc mesenteroides*. *Journal of General Microbiology, 138*, 2725–2731.

Hemme, D., & Foucaud-Scheuneman, C. (2004). *Leuconostoc*, characteristics, use in dairy technology and prospects in functional foods. *International Dairy Journal, 14*, 467–494.

Heng, N. C. K., & Tagg, J. R. (2006). What's in a name? Class distinction for bacteriocins. *Nature Reviews Microbiology, 4*, in press.

Heng, N. C. K., Wescombe, P. A., Burton, J. P., Jack, R. W., & Tagg, J. R. (2007). The diversity of bacteriocins in Gram-positive bacteria. In M. A. Riley, & M. A. Chavan (Eds.), *Bacteriocins ecology and evolution* (pp. 45-83). Berlin: Springer.

Herreros, M. A., Sandoval, H., González, L., Castro, J. M., Fresno, J. M. & Tornadijo. M. E. (2005). Antimicrobial activity and antibiotic resistance of lactic acid bacteria isolated from Armada cheese (a Spanish goats' milk cheese). *Food Microbiology, 22*, 455–459.

Holt, S. M., & Ricciardi, E. C. (2001). Occurrence and expression of β-galactosidase in dextran-producing *Leuconostoc* species. *Biotechnology Letters, 23*, 1147-1149.

Hutkins, R. W. (2006). Wine fermentation. In R. W. Hutkins (Eds.), *Microbiology and technology of fermented foods* (pp. 349-396). Iowa: Blackwell Publishing.

Jasniewski, J., Cailliez-Grimal, C., Younsi, M., Millière, J. B., & Revol-Junelles, A. M. (2008). Fluorescence anisotropy analysis of the mechanism of action of mesenterocin 52A speculations on antimicrobial mechanism. *Applied Microbial and Cell Physiology, 81*, 339–347.

Jófre, M. L., Sakurada Z. A., Ulloa F. T. M., Hormázabal O. J. C., Godoy M. V., Fernández O. J. et al. (2006). Infección por *Leuconostoc* en pacientes com síndrome de intestino corto, nutrición parenteral y alimentación enteral continua. *Revista Chilena Infectologia, 23*, 340-345.

Kekkonen, R. A., Kajasto, E., Miettinen, M., Veckman, V., Korpela, R., & Julkunen, I. (2008). Probiotic *Leuconostoc mesenteroides* ssp. *cremoris* and *Streptococcus thermophilus* induce IL-12 and IFN-γ production. *World Journal of Gastroenterology, 14*, 1192-1203.

Kikuchi, K., Totsuka, K., Shimizu, K., Yoshida, K., Kobayashi, M., Tomonaga, O., et al. (1994). Microbiological and clinical studies of vancomycin resistant *Leuconostoc* spp. and *Pediococcus* spp. isolated from septicemia patients. *Kansenshogaku Zasshi, 68*, 1084–1092.

Kjos, M. (2011). Target recognition, resistance, immunity and genome mining of class II bacteriocins from Gram-positive bacteria. *Microbiology, 157*, 3256–3267.

Klaenhammer, T. R. (1993). Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiology Reviews, 12*, 39–86.

Kostinek, M., Specht, I., Edward, V. A., Pinto, C., Egounlety, M., Sossa, C., et al. (2007). Characterization and biochemical properties of predominant lactic acid bacteria from fermenting cassava for selection as starter cultures. *International Journal of Food Microbiology, 114*, 342-351.

Kotzampassi, K., Giamarellos-Bourboulis, E. J., Voudouris, A., Kazamias, P., & Eleftheriadis, E. (2006). Benefits of a synbiotic formula (Synbiotic 2000Forte®) in critically ill trauma patients: early results of a randomized controlled trial. *World Journal of Surgery, 30*, 1848-1855.

Kumar, A., Kumar, M., Ghosh, M., & Ganguli, A. (2013). Modeling *in vitro* cholesterol reduction in relation to growth of probiotic *Lactobacillus casei. Microbiology and Immunology, 57*, 100-110.

Larsen, N., Vogensen, F. K., Gøbel, R., Michaelsen, K. F., Al-Soud, W. A., Sørensen, S. J., et al. (2011). Predominant genera of fecal microbiota in children with atopic dermatitis are not altered by intake of probiotic bacteria *Lactobacillus acidophilus* NCFM and *Bifidobacterium animalis* subsp. *lactis* Bi-07. *FEMS Microbiology Ecology, 75*, 482-496.

Lee, M. R., Huang, Y. T., Lee, P. I., Liao, C. H., Lai, C. C., Lee, L. N., et al. (2011). Healthcare-associated bacteraemia caused by *Leuconostoc* species at a university hospital in Taiwan between 1995 and 2008. *Journal of Hospital Infection, 78*, 45-49.

Lei, V., Amoa-Awua, W. K. A., Brimer, L. (1999). Degradation of cyanogenic glycosides by *Lactobacillus plantarum* strains from spontaneous cassava fermentation and other microorganisms. *International Journal of Food Microbiology, 53*, 169-184.

Leistner, L. (2000). Basic aspects of food preservation by hurdle technology. *International Journal of Food Microbiology, 55*, 181–186.

Leistner, L., & Gorris, L. G. M. (1995). Food preservation by hurdle technology. *Food Science Technology, 6*, 41–46.

Limonet, M., Revol-Junelles, A. M., Cailliez-Grimal, C., & Millière, J. B. (2004). Synergistic mode of action of mesenterocins 52A and 52B produced by *Leuconostoc mesenteroides* subsp. *mesenteroides* FR 52. *Current Microbiology, 48*, 204-207.

Linares, D. M., Martín, M. C., Ladero, V., Alvarez, M. A., & Fernández, M. (2011). Biogenic amines in dairy products. *Critical Reviews in Food Science and Nutrition, 51*, 691–703.

Ljungh, A., & Wadström, T. (2006). Lactic acid bacteria as probiotics. *Current Issues of Intestinal and Microbiology, 7*, 73–90.

Mainville, I., Arcand, Y., & Farnworth, E. R. (2005). A dynamic model that simulates the human upper gastrointestinal tract for the study of probiotics. *International Journal of Food Microbiology, 99*, 287-296.

Mäki, M. (2004). Lactic acid bacteria in vegetable fermentations. In S. Salminen, A. Von Wright, & A. Ouwehand (Eds.), *Lactic acid bacteria microbiological and functional aspects* (pp.419-430). New York: Marcel Dekker.

Masuda, Y., Ono, H., Kitagawa, H., Ito, H., Mu, F., Sawa, N., et al. (2011). Identification and characterization of leucocyclicin Q, a novel cyclic bacteriocin produced by *Leuconostoc mesenteroides* TK41401. *Applied and Environmental Microbiology, 77*, 8164–8170.

Meneghin, S. P., Reis, F. C., Almeida, P. G. de, & Ceccato-Antonini, C. R. (2008). Chlorine dioxide against bacteria and yeasts from the alcoholic fermentation. *Brazilian Journal of Microbiology, 39*, 337-343.

Mesas, J. M., Rodríguez, M. C., & Alegre, M. T. (2011). Characterization of lactic acid bacteria from musts and wines of three consecutive vintages of Ribeira Sacra. *Letters in Applied Microbiology, 52*, 258-268.

Metaxopoulos, J., Mataragas, M., & Drosinos, E. H. (2002). Microbial interaction in cooked cured meat products under vacuum or modified atmosphere at 4^{o}C. *Journal of Applied Microbiology, 93*, 363–373.

Monteagudo-Mera, A., Caro, I., Rodriguez-Aparicio, L. B., Rua, J., Ferrero, M. A., & Garcia-Armesto, M. R. (2011). Characterization of certain bacterial strains for potential use as starter or probiotic cultures in dairy products. *Journal of Food Protection, 74*, 1379-1386.

Montersino, S., Prieto, A., Muñoz, R., De Las Rivas, B. (2008). Evaluation of exopolysaccharide production by *Leuconostoc mesenteroides* strains isolated from wine. *Journal of Food Science, 73*, 196-199.

Moreno-Arribas, M. V., & Polo, C. M. (2008). Occurrence of lactic acid bacteria and biogenic amines in biologically aged wines. *Food Microbiology, 25*, 875-881.

Muñoz-Atienza, E., Gómez-Sala, B., Araújo, C., Campanero, C., Del Campo, R., Hernández, P. E. et al. (2013). Antimicrobial activity, antibiotic susceptibility and virulence factors of lactic acid bacteria of aquatic origin intended for use as probiotics in aquaculture. *BMC Microbiology, 13*, 1-22.

Nakamura, S., Kuda, T., An, C., Kanno, T., Takahashi, H., & Kimura, B. (2012). Inhibitory effects of *Leuconostoc mesenteroides* 1RM3 isolated from narezushi, a fermented fish with rice, on *Listeria monocytogenes* infection to Caco-2 cells and A/J mice. *Anaerobe, 18*, 19-24.

Nieto-Arribas, P., Seseña, S., Poveda, J. M., Palop, L., & Cabezas, L. (2010). Genotypic and technological characterization of *Leuconostoc* isolates to be used as adjunct starters in Manchego cheese manufacture. *Food Microbiology, 27*, 85-93.

Obilie, E. M., Tano-Debrah, K., & Amoa-Awua, W. K. (2004). Souring and breakdown of cyanogenic glucosides during the processing of cassava into akyeke. *International Journal of Food Microbiology, 93*, 115-121.

Oliva-Neto, P. de, & Yokoya, F. (1998). Effect of 3,4,4'-trichlorocarbanilide on growth of lactic acid bacteria contaminants in alcoholic fermentation. *Bioresource Technology, 63*, 17-21.

Osmanagaoglu, O., & Kiran, F. (2011). Evidence for a chromosomally determined mesenterocin, a bacteriocin produced by *Leuconostoc mesenteroides* subsp. *mesenteroides* OZ. *Journal of Basic Microbiology, 51*, 279–288.

Ouwehand, A. C., & Vesterlund, S. (2004). Antimicrobial components from lactic acid bacteria. In S. Salminen, A. Von Wright, & A. Ouwehand (Eds.), *Lactic acid bacteria microbiological and functional aspects* (pp.375-396). New York: Marcel Dekker.

Ouwehand, A. C., Kirjavainen, P. V., Grönlund, M. M., Isolauri, E., & Salminen, S. J. (1999). Adhesion of probiotic micro-organisms to intestinal mucus. *International Dairy Journal, 9*, 623-630.

Papathanasopoulos, M. A., Revol-Junelles, A. M., Lefebvre, G., Le Caer, J. P., Von Holy, A., & Hastings, J. W. (1997). Multiple bacteriocin production by *Leuconostoc mesenteroides* TA33a, and other *Leuconostoc*/*Weisella* strains. *Current Microbiology, 35*, 331–335.

Pasteris, S. E., Roig Babot, G., Otero, M. C., Bühler, M. I., & Nader-Macías, M. E. (2009). Beneficial properties of lactic acid bacteria isolated from a *Rana catesbeiana* hatchery. *Aquaculture Research, 40*, 1605-1615.

Pérez-Sánchez, T., Balcázar, J. L., Merrifield, D. L., Carnevali, O., Gioacchini, G., De Blas, I., et al. (2011). Expression of immune-related genes in rainbow trout (*Oncorhynchus mykiss*) induced by probiotic bacteria during *Lactococcus garvieae* infection. *Fish and Shellfish Immunology, 31*, 196-201.

Petri, A., Pfannebecker, J., Fröhlich, J., & König, H. (2013). Fast identification of wine related lactic acid bacteria by multiplex PCR. *Food Microbiology, 33*, 48-54.

Pircher, A., Bauer, F., & Paulsen, P. (2007). Formation of cadaverine, histamine, putrescine and tyramine by bacteria isolated from meat, fermented sausages and cheeses. *European Food Research and Technology, 226*, 225-231.

Raghavendra, P., & Halami, P. M. (2009). Screening, selection and characterization of phytic acid degrading lactic acid bacteria from chicken intestine. *International Journal of Food Microbiology, 133*, 129-134.

Rai, A. K., Tamang, J. P., Palni, U. (2010). Microbiological studies of ethnic meat products of the Eastern Himalayas. *Meat Science, 85*, 560-567.

Rani, P. S., & Agrawal, R. (2008). Effect on cellular membrane fatty acids in the stressed cells of *Leuconostoc mesenteroides*: A native probiotic lactic acid bacteria. *Food Biotechnology, 22*, 47-63.

Rani, S. (2008). *Isolation and characterization of a native isolate of Leuconostoc for functional attributes.* 292p. [Thesis (Doctor Degree) – Departament of Food Microbiology, Central Food Technological Research Institute, University of Mysore, India].

Ratti, R. P., Gomes, B. R., Martinez, R. C. R., Souza, V. M., & De Martinis, E. C. P. (2010). Elongated cells of *Listeria monocytogenes* in biofilms in the presence of sucrose and bacteriocin-producing *Leuconostoc mesenteroides* A11. *Ciência e Tecnologia de Alimentos, 30*, 1011-1016.

Ray, B. (2004). *Fundamental food microbiology*. (3rd ed.). New York: CRC Press.

Rayes, N., Seehofer, D., Theruvath, T., Mogl, M., Langrehr, J. M., Nüssler, N. C., et al. (2007). Effect of enteral nutrition and synbiotics on bacterial infection rates after pylorus-preserving pancreatoduodenectomy: A randomized, double-blind trial. *Annals of Surgery, 246*, 36-41.

Rayes, N., Seehofer, D., Theruvath, T., Schillerb, R. A., Langrehr, J. M., Jonas, S., et al. (2005). Supply of pre- and probiotics reduces bacterial infection rates after liver transplantation - A randomized, double-blind trial. *American Journal of Transplantation, 5*, 125-130.

Rayes, N., Seehofera, D., & Seehofera, D. (2009). Prebiotics, probiotics, synbiotics in surgery - Are they only trendy, truly effective or even dangerous? *Langenbecks Arch Surg, 394*, 547-555.

Reis, J. A., Casarotti, S. N., Paula, A. T., & Penna, A. L. B. (2011). *Probióticos e seus efeitos terapêuticos na saúde humana.* São José do Rio Preto: Cultura Acadêmica.

Reis, J. A., Paula, A. T., Casarotti, S. N., & Penna, A. L. B. (2012). Lactic acid bacteria antimicrobial compounds: characteristics and applications. *Food Engineer Reviews, 4*, 124-140.

Revol-Junelles, A. M., Mathis, R., Krier, F., Fleury, Y., Delfour, A., & Lefebvre, G. (1996). *Leuconostoc mesenteroides* subsp. *mesenteroides* FR52 synthesizes two distinct bacteriocins. *Letters in Applied Microbiology, 23*, 120-124.

Riley, M. A., & Chavan, M. A. (2007). *Bacteriocins ecology and evolution.* Berlin: Springer.

Rojo-Bezares, B., Sáenz, Y., Poeta, P., Zarazaga, M., Ruiz-Larrea, F., & Torres, C. (2006). Assessment of antibiotic susceptibility within lactic acid bacteria strains isolated from wine. *International Journal of Food Microbiology, 111*, 234-240.

Russo, P., López, P., Capozzi, V., De Palencia, P. F., Dueñas, M. T., Spano, G., et al. (2012). Beta-glucans improve growth, viability and colonization of probiotic microorganisms. *International Journal of Molecular Sciences, 13*, 6026-6039.

Ryu, E. H., & Chang, H. C. (2013). *In vitro* study of potentially probiotic lactic acid bacteria strains isolated from *kimchi*. *Annals of Microbiology*, in press.

Saarela, M., Mogensen, G, Fondén, R., Mättö, J., & Mattila-Sandholm, T. (2000). Probiotic bacteria: safety, functional and technological properties. *Journal of Biotechnology, 84*, 197-215.

Samelis, J., & Georgiadou, K. G. (2000). The microbial association of Greek taverna sausage stored at 4 and 10 °C in air, vacuum or 100% carbon dioxide, and its spoilage potential. *Journal of Applied Microbiology, 88*, 58–68.

Sanz, Y., Sánchez, E., Marzotto, M., Calabuig, M., Torriani, S., & Dellaglio, F. (2007). Differences in faecal bacterial communities in coeliac and healthy children as detected by PCR and denaturing gradient gel electrophoresis. *FEMS Immunology and Medical Microbiology, 51*, 562-568.

Seo, B. J., Rather, I. A., Kumar, V. J. R., Choi, U. H., Moon, M. R., Lim, J. H., et al. (2012). Evaluation of *Leuconostoc mesenteroides* YML003 as a probiotic against low-pathogenic avian influenza (H9N2) virus in chickens. *Journal of Applied Microbiology, 113*, 163-171.

Shobharani, P., & Agrawal, R. (2011). A potent probiotic strain from cheddar cheese. *Indian Journal of Microbiology, 51*, 251-258.

Singh, K., Kallali, B., Kumar, A., & Thaker, V. (2011). Probiotics: A review. *Asian Pacific Journal of Tropical Biomedicine, suppl. 2*, S287-S290.

Sip, A., Więckowicz, M., Olejnik-Schmidt, A., & Grajek, W. (2012). Anti-*Listeria* activity of lactic acid bacteria isolated from golka, a regional cheese produced in Poland. *Food Control, 26*, 117-124.

Stiles, M. E., & Holzapfel, W. H. (1997). Lactic acid bacteria of foods and their current taxonomy. *International Journal of Food Microbiology, 36*, 1-29.

Strausbaugh, C. A., & Gillen, A. M. (2008). Bacteria and yeast associated with sugar beet root rot at harvestin the intermountain west. *Plant Disease, 92*, 357-363.

Thapa, N., Pal, J., & Tamang, J. P. (2006). Phenotypic identification and technological properties of lactic acid bacteria isolated from traditionally processed fish products of the Eastern Himalayas. *International Journal of Food Microbiology, 107*, 33-38.

Todorov S. D., & Dicks L. M. T. (2008). Evaluation of lactic acid bacteria from kefir, molasses and olive brine as possible probiotics based on physiological properties. *Annals of Microbiology, 58*, 661-670.

Todorov, S. D., & Dicks, L. M. T. (2004). Characterization of mesentericin ST99, a bacteriocin produced by *Leuconostoc mesenteroides* subsp. *dextranicum* ST99 isolated from boza. *Journal of Industrial Microbiology and Biotechnology, 31*, 323–329.

Todorov, S. D., & Dicks, L. M. T. (2005). Characterization of bacteriocins produced by lactic acid bacteria isolated from spoiled black olives. *Journal of Basic Microbiology, 45*, 312–322.

Tok, D., Ilkgul, O., Bengmark, S., Aydede, H., Erhan, Y., Taneli, F., et al. (2007). Pretreatment with pro- and synbiotics reduces peritonitis-induced acute lung injury in rats. *Journal of Trauma - Injury, Infection and Critical Care, 62*, 880-885.

Trias, R., Badosa, E., Montesinos, E., & Bañeras, L. (2008). Bioprotective *Leuconostoc* strains against *Listeria monocytogenes* in fresh fruits and vegetables. *International Journal of Food Microbiology, 127*, 91-98.

Van Der Mei, H. C., Bos, R., & Busscher, H. J. (1998). A reference guide to microbial cell surface hydrophobicity based on contact angles. *Colloids and Surfaces B: Biointerfaces, 11*, 213-221.

Van Reenen, C. A., & Dicks, L. M. T. (2011). Horizontal gene transfer amongst probiotic lactic acid bacteria and other intestinal microbiota: What are the possibilities? A review. *Archives of Microbiology, 193*, 157-168.

Vay, C., Cittadini, R., Barberis, C., Rodríguez, C. H., Martínez, H. P., Genero, F., et al. (2007). Antimicrobial susceptibility of non-enterococcal intrinsic glycopeptide-resistant Gram-positive organisms. *Diagnostic Microbiology and Infectious Disease, 57*, 183-188.

Vázquez, E., Carazo, I., Martin, A., Lozano, C., Cuesta, I., & Pagola, C. (1998). Infective endocarditis due to *Leuconostoc mesenteroides*. *Enfermedades Infecciosas y Microbiologia Clinica, 16*, 237-238.

Vélez, M. P., Hermans, K., Verhoeven, T. L. A., Lebeer, S. E., Vanderleyden, J., & De Keersmaecker, S. C. J. (2007). Identification and characterization of starter lactic acid bacteria and probiotics from Columbian dairy products. *Journal of Applied Microbiology, 103*, 666–674.

Vendrell, D., Luis Balcázar, J., De Blas, I., Ruiz-Zarzuela, I., Gironés, O., & Luis Múzquiz, J. (2008). Protection of rainbow trout (*Oncorhynchus mykiss*) from lactococcosis by probiotic bacteria. *Comparative Immunology, Microbiology and Infectious Diseases, 31*, 337-345.

Vermeiren, L., Devlieghere, F., & Debevere, J. (2006). Co-culture experiments demonstrate the usefulness of *Lactobacillus sakei* 10A to prolong the shelf-life of a model cooked ham. *International Journal of Food Microbiology, 108*, 68-77.

Vidyalaxme, B., Rovetto, A., Grau, R., & Agrawal, R. (2012). Synergistic effects of probiotic *Leuconostoc mesenteroides* and *Bacillus subtilis* in malted ragi (*Eleucine corocana*) food for antagonistic activity against *V. cholerae* and other beneficial properties. *Journal of Food Science and Technology*, in press.

Wenocur, H. S., Smith, M. A., Vellozzi, E. M., Shapiro, J., & Isenberg, H. D. (1988). Odontogenic infection secondary to *Leuconostoc* species. *Journal of Clinical Microbiology, 26*, 1893–1894.

Wulijideligen, Asahina, T., Hara, K., Arakawa, K., Nakano, H., & Miyamoto, T. (2012). Production of bacteriocin by *Leuconostoc mesenteroides* 406 isolated from Mongolian fermented mare's milk, airag. *Animal Science Journal,* in press.

Xiraphi, N., Georgalaki, M., Rantsiou, K., Cocolin, L., Tsakalidou, E. & Drosinos, E. H. (2008). Purification and characterization of a bacteriocin produced by *Leuconostoc mesenteroides* E131. *Meat Science, 80,* 194-203.

Xu, H., Jeong, H. S., Lee, H. Y., & Ahn, J. (2009). Assessment of cell surface properties and adhesionpotential of selected probiotic strains. *Letters in Applied Microbiology, 49*, 434–442.

Yurdugül, S., & Bozoglu, F., (2002). Studies on an inhibitor produced by lactic acid bacteria of wines on the control of malolactic fermentation. *European Food Research and Technology, 215*, 38–41.

Zotta, T., Ricciardi, A., & Parente, E. (2007). Enzymatic activities of lactic acid bacteria isolated from Cornetto di Matera sourdoughs. *International Journal of Food Microbiology, 115*, 165-172.

Zouhir, A., Hammami, R., Fliss, I., & Hamidaet, J. B. (2010). A new structure-based classification of gram-positive bacteriocins. *The Protein Journal, 29*, 432-439.

Capítulo II

Leuconostoc mesenteroides SJRP55: a potential probiotic strain isolated from Brazilian water buffalo mozzarela cheese

Este Capítulo será submetido à Revista *Archives of Microbiology*

Leuconostoc mesenteroides SJRP55: potential probiotic strain isolated from Brazilian water buffalo mozzarela cheese

Aline T. de Paula, Ana Beatriz Jeronymo-Ceneviva, Luana F. Silva, Svetolav D. Todorov, Bernadette D. G. M. Franco, Ana Lúcia B. Penna

Aline de Paula · Ana Beatriz Jeronymo-Ceneviva · Luana Silva · Ana Lúcia Penna (e-mail)

UNESP - Sao Paulo State University, Food Engineering and Technology Department, Rua Cristovão Colombo, 2265, 15.054-000, +55 17 3221-2266, São José do Rio Preto, Brazil. E-mail: analucia@ibilce.unesp.br

Svestolav Todorov · Bernadette Franco

USP - Sao Paulo University, Faculty of Pharmaceutical Sciences, Department of Food Science and Experimental Nutrition, Av. Prof. Lineu Prestes, 580, bl. 13B, 05508-000, Sao Paulo, Brazil

Abstract

The probiotic potential of *Leuconostoc mesenteroides* subsp. *mesenteroides* SJRP55 isolated from water buffalo mozzarella cheese was evaluated. The microorganism presented resistance to stressful conditions that simulated the gastrointestinal tract; and to the best of our knowledge *Leuconostoc mesenteroides* SJRP55 was the first of this specie with ability to deconjugate bile salts. Tolerance to NaCl was temperature dependent, as well the results obtained by aggregation capacity. The strain presented good adhesion properties, *β*–galactosidase activity, viability in fermented milk during storage, non-active against *Streptococcus thermophilus* and sensible to most of the tested antibiotics. Some analgesic medications inhibited the growth of the strain. *Leuconostoc mesenteroides* SJRP55 exhibited *in vitro* probiotic potential, and it can be better characterized through future *in vivo* tests. This bacterium is a potential candidate for the application as a probiotic strain, which could be used by industries in the manufacture of functional milk-based food products.

Keywords: lactic acid bacteria, therapeutical characteristics, aggregation, bile salts, adhesion properties, fermented milk.

Introduction

The consumption of functional dairy products that provide health benefits has increased significantly in the last few years, and consequently, the industry has begun looking for strains with probiotic characteristics for future application in functional foods. Probiotics are defined as live, non-pathogenic microorganisms that, when administrated in adequate amounts, confer a health benefit to the consumer (FAO/WHO 2002).

The literature describes countless benefits of probiotic cultures. They have been found to enhance the host's immune response, to alleviate symptoms of lactose intolerance, to produce certain vitamins, to be useful in the treatment of many types of diarrhea, to compete with and inhibit pathogenic microorganisms, to reduce cholesterol, the risk of colon cancer, and allergic symptoms, to suppress infection by *Helicobacter pylori*, to improve oral health and to influence the course of critically ill patients, among other benefits (Adams 2010; Reis et al. 2011; Singh et al. 2011; Amara and Shibl 2013; de Azevedo et al. 2013). However, some of the mechanisms by which probiotic strains exert beneficial effects are largely unknown or not well understood (Amara and Shibl 2013).

Some epidemiological studies have shown the beneficial effects of *Leuconostoc* strains. The reduction of acute diarrhea in children was confirmed after the children were fed with Indian Dahi, a traditional Indian fermented milk containing *Lactococcus lactis*, *Lactococcus lactis* subsp. *cremoris* and, *Lc. mesentereoides* subsp. *cremoris* (Agarwal and Bhasin 2002). Use of pre- and probiotics have been found to reduce bacterial infection rates after liver transplantation and in patients who have undergone other high-risk surgeries (Rayes et al. 2005; Rayes et al. 2009).

To be considered as a probiotic culture, the microorganism must be safe (non-pathogenic, absent of virulence genes and antibiotics resistance, and present genetic stability); it must possess technological food qualities (viable during the storage period, bacteriophage resistance, the ability to be produced in large scale); it must survive the stress condition(s) of GIT; it must be able to adhere and colonize the intestinal cells, and it must present therapeutic benefits. All of

these descriptions should be validated and documented, and the replicate results found in *in vitro* trials should be confirmed through *in vivo* assays (Reis et al. 2011; Fontana et al. 2013).

In an attempt to promote health and to ensure the treatment or management of diseases, probiotics are present in many types of foods (Amara and Shibl 2013). The use of lactic acid bacteria (LAB) as probiotic cultures by dairy industries has become more frequent, and it is acceptable to consumers due of the production of flavor, aroma by the culture (Shiby and Mishra 2013). Moreover, when present in dairy products, these microorganisms can produce lactic acid and other antimicrobial compounds, such as bacteriocins, which can inhibit undesirable microorganisms, thus extending the shelf-life of products, and promoting therapeutic, sensory, and technological food benefits (Kos et al. 2007).

Bacteria belonging to the genera *Leuconostoc* are heterofermentative LAB isolated mainly from vegetables, cereal, silage, fruits, wine, fish, meat, and dairy products. However, this microorganism may be involved in the deterioration of some products and, in rare cases, it may also be involved in diseases in immunocompromised patients (Lee et al. 2011).

Some studies on the evaluation of *Lc. mesenteroides* subsp. *mesenteroides* as a potential probiotic culture are still preliminary; however, researches studies have proven the probiotic characteristics of this microorganism (Agarwal and Bhasin 2002; Aswathy et al. 2008; Tamang et al. 2009; Shobharani and Agrawal 2011; Allameh et al. 2012; Seo et al. 2012).

The objective of this study was to evaluate the characteristics of a potential probiotic strain *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 isolated from water buffalo mozzarella cheese, including the determination of its viability in co-culture with a commercial starter microorganism in fermented milk during the storage condition.

Materials and methods

Culture media and incubation conditions

Lc. mesenteroides subsp. *mesenteroides* SJRP55, a bacteriocinogenic strain (Paula et al. 2012) was isolated from water buffalo mozzarella cheese and identified by whole 16S rDNA gene sequencing (Silva 2010). The strain was cultured in MRS broth (Difco laboratories, Detroit, MI, USA) at 30 ^{o}C and stored at −80 ^{o}C with 20 % (v/v) glycerol.

Tolerance to pH, bile and NaCl

A static *in vitro* model to determine the transit tolerance through simulated gastric juice and the ability of the strain to grow in the presence of bile and NaCl was carried out Todorov et al. (2008). *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 was cultured overnight in MRS broth adjusted with different pH values (3.0, 4.0, 5.0, 6.0, 7.0, 9.0, 11.0 and 13.0), oxbile (0.2, 0.4, 0.6, 1.0, 2.0 and 3.0 % [w/v]: Sigma-Aldrich, St Louis, MO, USA) and NaCl (0, 0.5, 1.0, 2.0, 3.0, 5.0 and 10.0 [w/v]: Synth, Diadema, São Paulo, Brazil). All tests were conducted in sterile flat-bottom 96-well micro titer plates (TPP, Trasadingen, Switzerland). Each well was filled with 180 µl of modified MRS broth (pH, oxbile and NaCl) and 20 µl of the culture (Optical density at 600 nm - $OD_{600\ nm}$ = 0.2). The strain was incubated at 37 ^{o}C for the pH and oxbile tests and at 5 ^{o}C, 30 ^{o}C and 37 ^{o}C for the NaCl test. These temperatures were chosen to simulate the optimum growth temperature (30 ºC), the natural human body temperature (37 ºC), and the refrigerated storage of dairy products (5 ºC). Every hour for 12 h, the $OD_{600\ nm}$ was recorded. Experiments were performed in triplicate.

Bile salts deconjugation

Bile salt hydrolase activity was evaluated according Kumar et al. (2013) with slight modifications. Ten microliters of overnight cultures were spotted onto two different modified MRS agar plates supplemented with 0.5 % (w/v) taurdoeoxycholic acid sodium salt (Sigma-Aldrich) or taurocholic acid sodium salt hydrate (Sigma-Aldrich), both with 0.37 g/l of calcium

chloride (Synth). The plates were incubated at 30 °C for 72 h. The strains displaying a white precipitation zone surrounding the colonies were considered to be positive.

Auto-aggregation and co-aggregation assays

The cells present in 10 ml of an overnight culture of the *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 and the tested cultures of *Enterococcus faecalis* ATCC 19443, *Lc. mesenteroides* subsp. *mesenteroides* UCV10CET (MRS broth, 30 °C) and *Listeria innocua* ATCC 33090 and *Listeria monocytogenes* ATCC 7644 (BHI broth [Difco], 30 °C) were washed with sterile saline solution (pH 6.5), harvested by centrifugation (7000 x *g*, 10 min, 20 °C), and diluted to $OD_{660\ nm}$ = 0.3 (Todorov et al. 2008). Cells were transferred (1 ml) to a 2-ml sterile Eppendorf tube, and the samples were incubated at 5 °C, 30 °C and 37 °C. These temperatures were chosen to simulate the optimum growth temperature (30 ºC), the natural human body temperature (37 ºC), and the refrigerated storage of dairy products (5 ºC). After 1 h, the cell suspension was centrifuged (300 x *g*, 2 min, 20 °C) and the $OD_{660\ nm}$ of the supernatant was determined. The percentage of auto-aggregation was calculated using the following formula: $[(OD_0 - OD_{60})/ OD_0] \times 100$, where OD_0 refers to initial OD and OD_{60} refers to the OD value measured after 60 min, respectively. The co-aggregation trials were performed with overnight cultures of the *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 in combination with the tested cultures of *Enterococcus faecalis* ATCC 19443, *Lc. mesenteroides* subsp. *mesenteroides* UCV10CET (MRS broth, 30 °C) and *Listeria innocua* ATCC 33090 and *Listeria monocytogenes* ATCC (BHI broth, 30 °C). The experimental protocol for the study of co-aggregation was the same used for auto-aggregation. The co-aggregation trials were performed in presence of *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 cells in combination with the tested strains cells (500 µl of SJRP55 culture and 500 µl of indicator strain in a sterile plastic cuvette). The percentage of co-aggregation was calculated based on the same formula used for the auto-aggregation analysis. Experiments were conducted in triplicate on two separate occasions.

Cell surface hydrophobicity

The ability of the cell surface to adhere to hydrophobic compounds was evaluated according to the method reported by Doyle and Rosenberg (1995). Cells of the *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 were harvested (6700 x *g*, 4 °C, 6 min) from overnight culture obtained in MRS at 30 °C, washed twice with phosphate buffer (0.1 mol/l), suspended in the same solution, and the $OD_{580\ nm}$ was measured. Cell suspension (1.5 ml) was added to 1.5 ml of n-hexadecane (Sigma-Aldrich) and vortexed for 2 min. The aqueous and organic phases were allowed to separate for 30 min at room temperature. An aliquot of 1 ml of the aqueous phase was removed to determine the $OD_{580\ nm}$. The percentage of hydrophobicity was calculated based on the same formula used for the auto-aggregation analysis. Experiments were conducted in triplicate.

Adherence to Caco-2 cells

The Caco-2 cell line, known as ATCC HTB-37 (Rio de Janeiro Cell Bank, Rio de Janeiro, Brazil), was routinely cultured (29-31 days) in Dulbelcco's modified Eagle's minimum medium (DMEM) (Sigma-Aldrich), supplemented with 20 % (v/v) heat-inactivated fetal bovine serum (Cultilab, Campinas, Brazil), a mixture of penicillin (100 U.I/ml) and streptomycin (100 µg/ml) solution (Sigma-Aldrich), and 1 % (v/v) non-essential amino acid solution (Sigma-Aldrich) at 37 °C in an atmosphere of 5 % CO_2 and 95 % air. The adhesion assay was performed as described by Argyri et al. (2013), with modifications. Caco-2 cells were seeded at a concentration of 10^5 cells per well into 24 well tissue culture plates (NEST) and incubated at 37 °C in 5 % CO_2 95 % air atmosphere (Thermo Fisher Scientific, Waltham, MA, USA) until the formation of a confluent monolayer was formed (15-17 days). One day before the performance of the adhesion assays, the medium was replaced, but without antibiotics. Before adhesion, the monolayer was washed once with phosphate-buffered saline (PBS, pH 7.4), to remove all traces of the medium. The *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 was grown overnight, until it reached the stationary phase in MRS at 30 °C, and it was then washed twice with sterile PBS. Subsequently, approximately 10^8 cfu/ml was transferred to post-confluent monolayers of Caco-

2 cells in the 24-well tissue culture plates and incubated at 37 °C in 5 % CO_2 95 % air atmosphere for 2 h. Cells were then washed at least three times with PBS, in order to remove both the non-adherent bacteria and the cells with adherent bacteria from each well with the addition of 1 ml of Triton X-100 (0.5 %, v/v) (Sigma-Aldrich). The suspension (1 ml) from each well was then transferred to a tube containing 9 ml of sterile saline, serially diluted, and plated on MRS agar in duplicate, in order to determine adhesion ability. Adherence (expressed as a percentage) was calculated using the ratio of the number of bacterial cells that remained attached to the total number of bacterial cells added initially to each well. The experiment was performed in triplicate on two separate occasions.

Enzymatic activity

Enzymatic activities were assayed using an API ZYM kit (BioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. For this experiment, it was used a 24 h-old culture of *Lc. mesenteroides* subsp. *mesenteroides* SJRP55, previously activated on MRS agar (Difco) at 30 °C.

Evaluation of survival of *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 in fermented milk

In order to study the viability of *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 and its bacteriocinogenic potential against the starter culture *Streptococcus thermophilus* TA040 (Danisco, Sassenage, France) in fermented milk, both strains were been cultured in reconstituted commercial skim milk (Molico®, Nestlé, Brazil) at 12 % (w/w) of total solids, and the following treatments were used for the tests: (I) *Streptococcus thermophilus* TA040 (St), (II) St + *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 (St+SJRP55); (III) *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 (SJRP55). Before the inoculation, the reconstituted milk samples (200 ml) were subject to heat treatment at 90 ^{o}C for 10 min in water bath (Marconi, Piracicaba, SP, Brazil), followed by a cooling step done at 10 ^{o}C and storage overnight at 4 ^{o}C. Experiments were performed with the initial levels of bacterial populations at 10^8 cfu/ml for both strains.

After inoculation, experimental milk samples were incubated at 37 ^{o}C in a thermostatically controlled water bath, monitored by Cinac (*Cinétique d'acidification*) system (Ysebaert, Frépillon, France) until the pH 4.6 was reached. After incubation, the fermented milks were cooled on ice bath for 1 h to reach 5 ^{o}C, and then they were manually agitated by use of a stainless steel perforated disk-rod that was moved upwards and downwards for 60 s, followed by dispensing the material into 50 ml polypropylene sterile flasks and stored at 4 ^{o}C. The viability of cells was evaluated at 1, 7, 14, 21 and 28 refrigerated storage days. Fermented milks were prepared in two independent assays, resulting in two trials for each type of fermented milk. The cell counts of LAB (*St. thermophilus* TA040 and *Lc. mesenteroides* subsp. *mesenteroides* SJRP55) were made in duplicate during the refrigerated storage. Homogenized samples (1.0 mL) were subject to serial decimal dilutions in 0.1 % saline solution and plated on M17 agar/37 °C for *St. thermophilus* TA040 and MRS agar supplemented with 1.5 % of oxbile/30 °C (Difco) for *Lc. mesenteroides* subsp. *mesenteroides* SJRP55, both of them were incubated for 48 h, according to Dave and Shah (1996). Colony forming units (cfu) were enumerated in plates containing 25 to 250 colonies, and cell concentration was expressed as log cfu/ml of fermented milk.

Medications and antibiotics

Resistance of *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 to drugs from different groups and selected antibiotics was tested according to Todorov et al. (2011). The drugs were purchased in a local drugstore (Sao Jose do Rio Preto, SP, Brazil) and solubilized in sterile water to achieve the desired concentration (Table 1). An 18 h-old culture of SJRP55 was inoculated into 20 ml of MRS soft agar (1.0 %, w/v; Difco) to a final concentration of 10^{6} cfu/ml. After solidification, the drugs were diluted separately in 5 ml of sterile distillated water and 10 µl was spotted onto the surface of the agar and incubated at 30 °C for 24 h. Inhibition zones around the spotted drug were checked, and those which presented inhibition zones that were larger than 2 mm in diameter were tested to determine the minimal inhibitory concentration (MIC). For this test, a serial twofold dilution of the drugs was prepared in sterile

water and 10 µl were spotted onto the surface of the 20 mL of MRS soft agar plates that had been previously inoculated with the *Lc. mesenteroides* subsp. *mesenteroides* SJRP55. The plates were incubated for 24 h at 30 °C and examined for the presence of inhibition zones around the spotted drug. Inhibition zones larger than 2 mm diameter were subjected to the determination of the minimal inhibition concentration (MIC). For this test, serial twofold dilutions of the drugs were prepared in sterile water and 10 µl spotted onto the surface of MRS soft agar plates, previously inoculated with *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 (10^6 cfu/ml). The MIC corresponds to the highest dilution that resulted in inhibition halos of at least 2 mm diameter.

The sensitivity to antibiotics (Table 2) was tested under the same conditions used to test the medications − the spots of drugs were replaced with antibiotics, which were applied using the disc diffusion test (Oxoid, Hampshire, England). The diameters of the inhibition zones surrounding the disks were measured in millimeters.

Results

Tolerance to pH, bile salts, NaCl

Lc. mesenteroides subsp. *mesenteroides* SJRP55 was more resistant in alkaline pH (9.0-11.0) than under other conditions (3.0-6.0; 13.0). The pH 13.0 resulted in a significant loss of viability. During the incubation period, the pHs 3.0-7.0 promoted a slight interference on the growth of the culture (Fig. 1). *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 grew well in all concentrations of bile salts tested (between 0.2 % and 3.0 %, Fig. 2). A white precipitation zone surrounding the colony showed the ability of *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 culture to deconjugate sodium taurodeoxycholic acid sodium salt and taurocholic acid.

The presence of different concentrations of NaCl and the incubation temperature tested were limiting factors for the growth of *Lc. mesenteroides* subsp. *mesenteroides* SJRP55, which has survived in all temperatures analyzed (5 ºC, 30 ºC, 37 ºC), but the growth was reduced,

mainly at 5 ºC (Fig. 3). Moreover, an increase in NaCl concentration resulted in a reduction in the growth and survival of the culture which has tolerated up to 5 % NaCl at 30 ºC and 37 ºC.

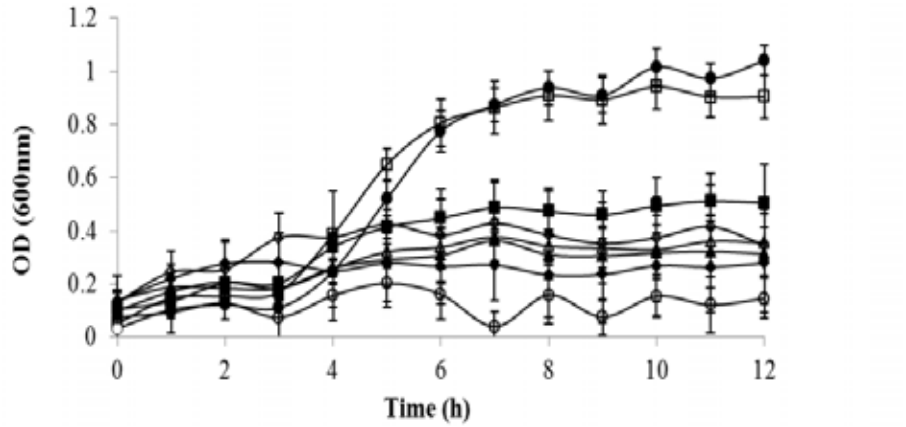


Fig. 1 Growth of *Leuconostoc mesenteroides* subsp. *mesenteroides* SJRP55 in MRS broth at pH levels adjusted from 3.0−13.0, shown as OD (600nm) measurements. The results are represented as an average of three readings. (♦) 3.0, (◊) 4.0, (▲) 5.0, (Δ) 6.0, (■) 7.0, (□) 9.0, (●) 11.0, (○) 13.0

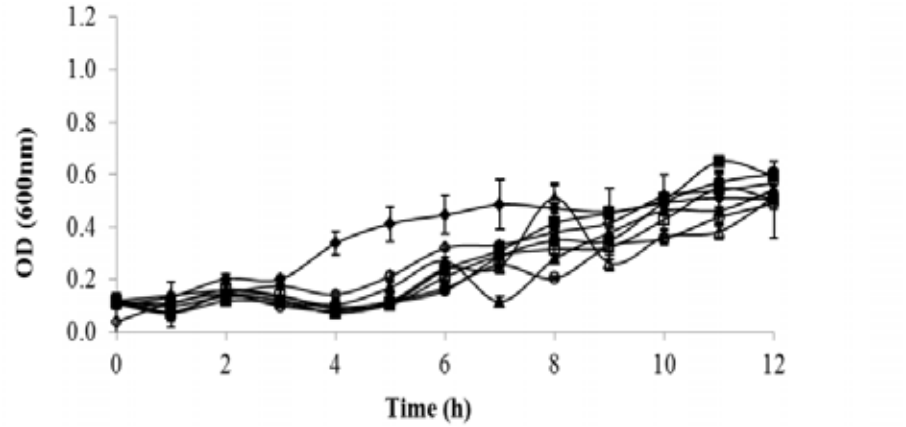


Fig. 2 Growth of *Leuconostoc mesenteroides* subsp. *mesenteroides* SJRP55 in MRS broth supplemented with 0–3.0 % bile salts, shown as OD (600nm) measurements. The results are represented as an average of three readings. (♦) 0, (◊) 0.2, (▲) 0.4, (Δ) 0.6, (■) 0.8, (□) 1.0, (●) 2.0, (○) 3.0%

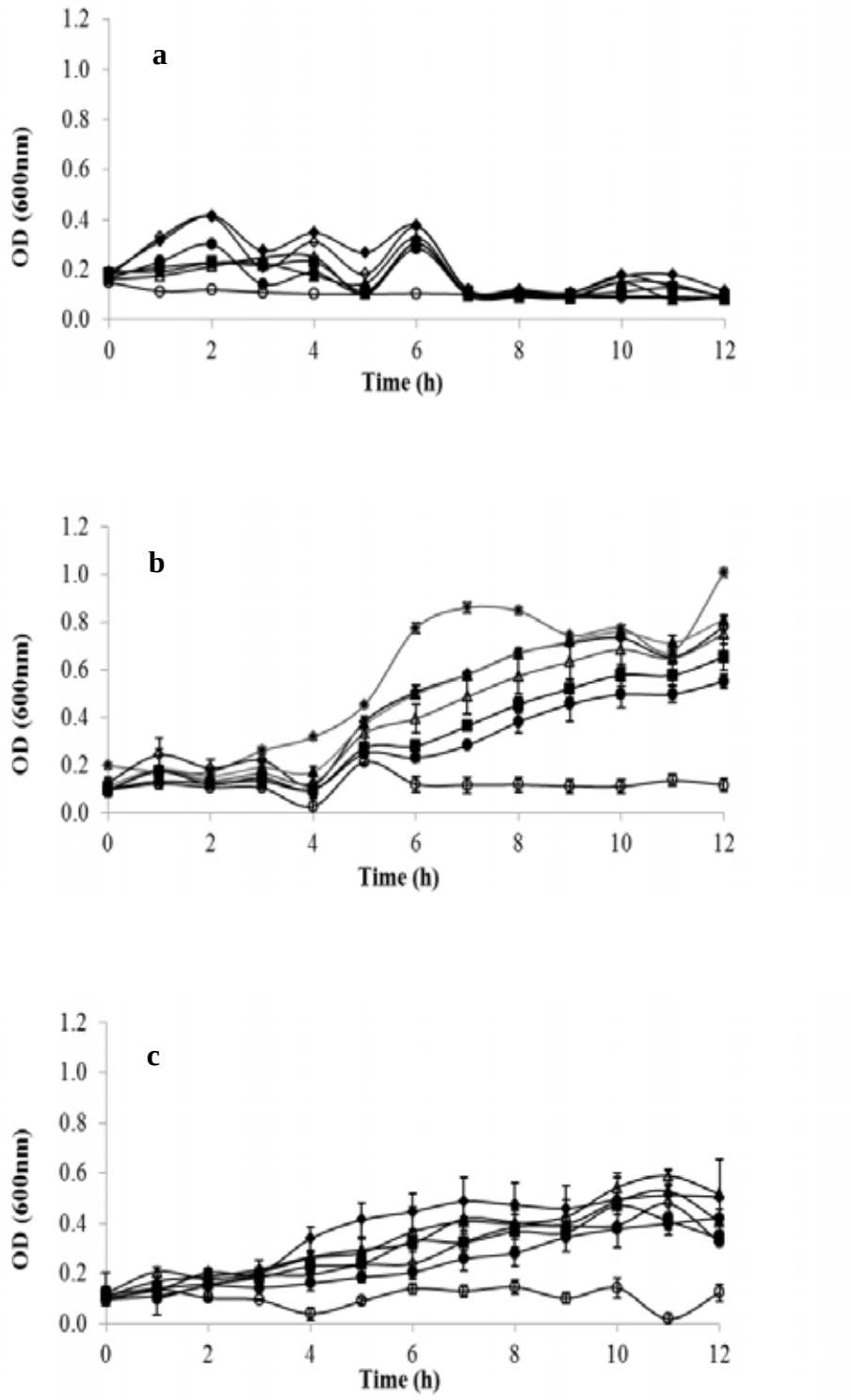


Fig. 3 Growth of *Leuconostoc mesenteroides* subsp. *mesenteroides* SJRP55 in MRS broth with 0 – 10·0 % of NaCl at 5 ºC (a), 30 ºC (b) and 37 ºC (c), shown as OD (660nm) measurements. The results are represented as an average of three readings. (♦) 0, (◊) 0.5, (▲) 1.0, (Δ) 2.0, (■) 3.0, (●) 5.0, (○) 10.0%

Auto-aggregation and co-aggregation assays

The highest auto-aggregation rates of *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 (85.64 %) was observed at 30 ºC (Fig. 4). When the tests were performed at 37 ºC and 5 ºC, similar auto-aggregation values were obtained (51.05 % and 51.67 %), respectively. In addition, *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 presented a greater ability to auto-aggregate than the indicator cultures at 30 ºC. Among the indicator cultures, the most auto-aggregative strain was *L. innocua* at 5 ºC (60.71 %) and at 30 ºC (80.52 %), and *Enteroccoccus faecalis* at 30 ºC (52.60 %). *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 was found to possess the ability to co-aggregate with the indicator strains tested. The highest value was with *Enteroccoccus faecalis* (90.37 %) at 30 ºC and the lowest was with *Lc. mesenteroides* subsp. *mesenteroides* UCV10CET (42.42%) at 5 ºC.

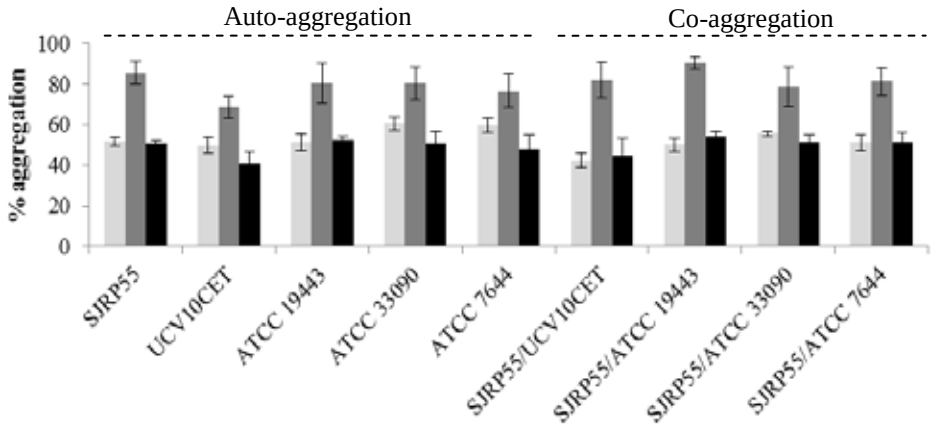


Fig. 4 Auto-aggregation and co-aggregation of *Leuconostoc mesenteroides* subsp. *mesenteroides* SJRP55, *Lc. mesenteroides* subsp. *mesenteroides* UCV10CET, *Enterococcus faecalis* ATCC 19443, *Listeria innocua* ATCC 33090, and *Listeria monocytogenes* ATCC 7644 at 5 ºC (light gray bar), at 30 ºC (dark gray bar), and at 37 ºC (black bar) expressed as percentage. Each result is represented as an average of three readings

Cell surface hydrophobicity

Lc. mesenteroides subsp. *mesenteroides* SJRP55 presented 59.12 % cell surface hydrophobicity, measured using the interaction with *n*-hexadecane to simulate the ability to adhere to the intestinal epithelium.

Adherence to Caco-2 cells

Lc. mesenteroides subsp. *mesenteroides* SJRP55 presented high adhesion (94.12 %) to Caco-2 cells.

Enzymatic activities

Lc. mesenteroides subsp. *mesenteroides* SJRP55 exhibited *β*–galactosidase activity. From the API ZYM kit test, it was also observed that *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 presented a weak enzymatic activity for leucine arylamidase, cystine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. It was not detected at studied conditions the production of proteinase, esterase, lipase and β-glucuronidase enzymes (data not shown).

Evaluation of co-survival of *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 and *Streptococcus thermophilus* TA040 in fermented milk

The viability of the tested LAB strains (*Lc. mesenteroides* subsp. *mesenteroides* SJRP55 and *St. thermophilus* TA040) was stable during the storage condition (Fig. 5). In the beginning of the tested period (1st day of analysis) *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 showed slow decrease in the cell counts, however, it recovered its growth during the refrigerated storage. When both strains (*Lc. mesenteroides* subsp. *mesenteroides* SJRP55 and *St. thermophilus* TA040) were been co-cultured, no inhibitory effect between them has been recorded.

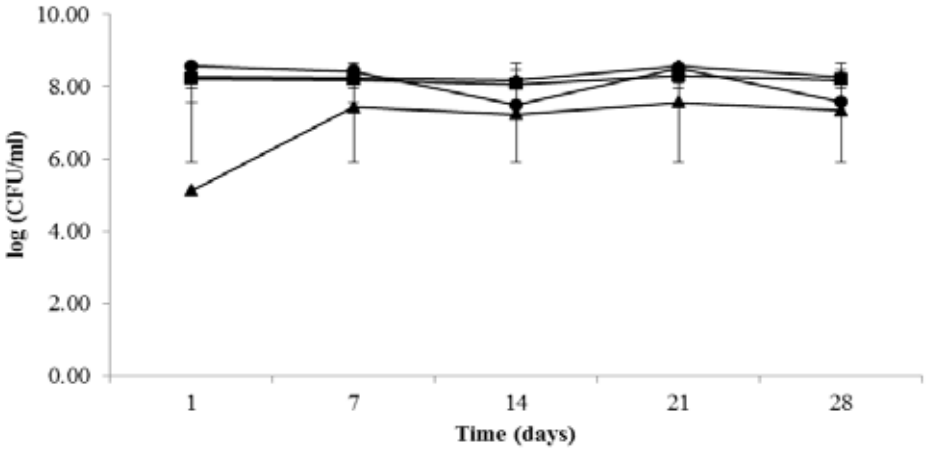


Fig. 5 Cell counts of *Streptococcus thermophilus* (St), *St. thermophilus* + *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 (St+SJRP55) and *Lc. mesenteroides* subsp. *mesenteroides* SJRP55(SJRP55) in fermented milk. The results were represented as two independent assays resulting in two trials for each type of milk. (♦) St, (■) St+SJRP55 (St), (▲) St+SJRP55 (SJRP55), (●) SJRP55

Medications and antibiotics

In order to address certain prerequirements of potential probiotic strain, the safety of the probiotic strain was evaluated among the 35 tested antibiotics. *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 was found to be resistant to only five antibiotics: nalidixic acid, sulfamethoxazole/trimethoprim, sulfonamide, teicoplanin and vancomycin (Table 1).

Table 1 Antibiotic susceptibility of *Leuconostoc mesenteroides* subsp. *mesenteroides* SJRP55

Antibiotic (µg per disc)	**Classification**[b]	**Clear zone diameter (mm)**
Amikacin 30	Aminoglycoside (inhibits protein synthesis)	19
Gentamicin 10		20
Kanamycin 30		16
Tobramycin 10		17
Amoxicillin/clavulanic acid 30	*β*-lactam (interfere with bacteria cell wall synthesis) / *β*-lactam (inhibits the *β*-lactamase)	23
Cefuroxime 30		28
Oxacillin 5 µg		16
Ampicillin 10	Penicillin / *β*-lactam (interfere in the bacteria cell wall synthesis)	24
Penicillin G 5		20
Bacitracin 10	Cyclic polypeptide (inhibits bacteria cell wall synthesis)	27
Cefaclor 30	Second-generation cephalosporin / *β*-lactam (interferes with bacterial cell wall synthesis)	16
Cefotaxim 30		22
Cefepime 30	Fourth-generation cephalosporin / *β*-lactam (interferes with bacterial cell wall synthesis)	16
Ceftazidim 30	Third-generation cephalosporin / *β*-lactam (interferes with bacterial cell wall synthesis)	11
Ceftiofur 30		22
Ceftriaxone 30		17
Ciprofloxacin 5	Fluoroquinolone (inhibits bacterial topoisomerase II)	13
Clarithromycin 15	Macrolide (inhibits protein synthesis)	25
Erythromycin E 10		26
Chloramphenicol 30	Chloramphenicol (prevents peptide bond formation – inhibits protein synthesis)	26
Doxycycline hydrochloridric 30	Tetracycline (inhibits protein synthesis)	31
Minocycline 30		32
Tetracycline 30		28
Linezolid 30		31
Florfenicol 30	Synthetic compound similar to chloramphenicol (prevent the linkage of peptides – inhibits protein synthesis	28
Imipenem 10	Carbapenem / *β*-lactam (interferes with bacterial cell wall synthesis)	21
Moxifloxacin 5	Fourth-generation synthetic fluoroquinolone (inhibiting DNA gyrase)	23
Nalidixic acid 30[a]	*Synthetic quinolone antibiotic (inhibiting DNA gyrase)*	*0*
Nitrofuranton 10	Nitrofuran derivative (nucleic acid inhibitor)	10
Ofloxacin 5	Lincosamide (inhibits protein synthesis)	19
Rifampicin 5	Semi-synthetic compound derivate from *Amycolatopsis rifamycinica*	24
Sulfamethoxazole/trimethoprim 25	*Sulfonamide (inhibits folate synthesis)*	*0*
Sulfonamide 300	*Inhibits folate synthesis*	*0*

Teicoplanin 30	*Glicopeptides / β-lactam (interferes with bacterial cell wall synthesis)*	*0*
Vancomycin 5		*0*

[a] Italic: antibiotics that did not affect the growth of *Lc. mesenteroides* subsp. *mesenteroides* SJRP55.
[b] Todorov et al. (2011), Todorov et al. (2012)

Among the 65 drugs tested, 12 affected the growth of *Lc. mesenteroides* subsp. *mesenteroides* SJRP55, and most of them were analgesic and anti-inflammatory medications containing ibuprofen, butylscopolamine, metamizole, caffeine, orphenadrine citrate and isometheptene mucate (Table 2). In addition, Cozaar (Losartan) was the only antihypertensive drug that affected the survival of the probiotic bacteria. The MICs found for the analgesic drugs. Dipirona sódica (3.0 mg/ml), Dorflex (1.8 mg/ml), Lisador (3.0 mg/ml) and Novalgina (6.25 mg/ml) were very low, comparing to the concentration commonly used by these drugs.

Table 2 Effect of medications on the growth of *Leuconostoc mesenteroides* subsp. *mesenteroides* SJRP55

Brazilian commercial name	**Concentration (mg/ml)**	**Active substance**	**Medication class**	**Inhibition zone (mm) [MIC (mg/ml)]**
Advil	40	Ibuprofen	Analgesic	23 [40.0]
Buscopan	2 50	Butylscopolamine Metamizole	Analgesic, antispasmodic	27 [12.5]
Cloridrato de prometazina	5	Promethazine hydrochloride	Antihistaminic	14 [5.0]
Cozaar	20	Losartan	Antihypertensive	13 [20.0]
Dipirona sódica	100	Metamizole	Analgesic	22 [3.0]
Dorflex	60 10 7	Metamizole Caffeine Orphenadrine citrate	Analgesic, anti-inflammatory and muscle relaxant	21 [1.8]
Ibuprofene Biogaran	40	Ibuprofen	Anti-inflammatory	23 [40.0]
Lisador	100 2 1	Metamizole Adifenine hydrochloride Promethazine hydrochloride	Analgesic, antispasmodic intestinal, uterine antispasmodic and antipyretic	19 [3.0]
Metotrexato	0.5	Methotrexate Sodium	Antimetabolic	35 [0.5]

Migraliv	0.2 70 20	Dihydroergotamine mesylate Metamizole Caffeine	Anti-migraine	29 [87.5]
Neosaldina	60 6 6	Metamizole Isometheptene mucate Anhydrous caffeine	Analgesic and antipyretic	27 [30.0]
Novalgina	100	Metamizole	Analgesic and antipyretic	25 [6.25]

The following commercial drugs have no effect on the growth of *Lc. mesenteroides* subsp. *mesenteroides* SJRP55: AAS adulto (acetylsalicylic acid, analgesic and antipyretic, at 100.0 mg/ml); Aldactone (spironolactone, diuretic, at 5.0 mg/ml); Ansiopax (*Piper methysticum*, anxiolytic, at 46.8 mg/ml); Biprofenid (ketoprofen, anti-inflammatory, analgesic, at 30.0 mg/ml); Cardilol (carvedilol, antianginal and antihypertensive, at 1.25 mg/ml); Celebra (celecoxib, anti-inflammatory, at 40.0 mg/ml); Cezarette (desogestrel, contraceptive, at 15.0 mcg/ml), Clonotril (clonazepam, anxiolytic, at 0.1 mg/ml); Cloridrato de fexofenadine (fexofenadine hydrochloride, anti-allergy, at 36.0 mg/ml); Cloridrato de propranolol (propranolol hydrochloride, antihypertensive, at 8.0 mg/ml); Cloxazolam (anxiolytic and sedative, at 0.4 mg/ml); Decongex plus (phenylephrine hydrochloride and brompheniramine maleate, decongestant of the upper respiratory tract**,** at 3.0 and 2.4 mg/ml); Diasec (loperamide hydrochloride, anti-diarrhoeal, at 0.4 mg/ml); Diovan amlo fix (valsartan, amlodipine, antihypertensive, at 32.0 and 1.0 mg/ml); Doliprane (paracetamol, analgesic, antipyretic, at 200.0 mg/ml); Dramin B6 (dimenhydrinate and pyridoxine hydrochloride, antiemetic, at 10.0 and 2.0 mg/ml); Ebastel (ebastine, antihistamynic, at 2.0 mg/ml); Enalprin (maleat enalapril, antihypertensive, at 4.0 mg/ml); Flamador (Ketoprofen, analgesic, anti-inflammatory, antirheumatic, at 10.0 mg/ml); Levoid (levothyroxine sodium, treatment of Thyroid problems, at 17.6 mcg/ml); LipLess (ciprofibrate, anti-hypertriglyceridaemia, at 20.0 mg/ml); Loratadina (loratadine, antihistamine, antiallergic, at 2.0 mg/ml); Maleato de enalapril (maleat enalapril, antihypertensive, at 2.0 mg/ml); Maracugina (*Passiflora alata*, *Erythrina mulungu*, *Crataegus oxyacantha*, neuro-sedative, at 5.0 and 2.5 mg/ml); Maxsulid (nimesulide beta-cyclodextrin, analgesyc, anti-inflammatory and antipyretic, at 80.0 mg/ml); Meloxicam (meloxicam, anti-inflammatory, at 3.0 mg/ml); Meticorten (prednisone, anti-inflammatory, at 4.0 mg/ml); Metiocolin B12 (DL-methionine and inositol, hepatoprotective, choline chloride and cobalamin, at 20.0, 10.0, 5.0 m mg/ml and 0.4 mcg/ml); Mioflex (paracetamol, carisoprodol, fenilbutazone, analgesic, anti-inflammatory, muscle relaxant, at 60.0, 30.0 and 15.0 mg/ml); Miosan (cyclobenzaprine hydrochloride, muscle relaxant, at 1.0 mg/ml); Motilium (domperidone, antiemetic, at 2.0 mg/ml); Omepramedi (omeprazole, proton pump inhibitor, at 4.0 mg/ml); Paracetamol (paracetamol, analgesic, antipyretic, at 150.0 mg/ml); Plasil (metoclopramide hydrochloride, antiemetic, at 2.0 mg/ml); Plaq (clopidogrel bisulfate, antihypertensive, at 15.0 mg/ml); Prelone (prednisolone, corticosteroid, at 1.0 mg/ml); Profenid enterico (ketoprofen, anti-inflammatory, analgesic, antipyretic, at 20.0 mg/ml); Primosiston (ethinyl estradiol and norethisterone acetate, antihemorrhagic, at 0.4 and 0.002 mg/ml); Resfenol (paracetamol, chlorpheniramine maleate and phenylephrine hydrochloride, analgesyc and antipyretic, at 80.0 and 0.8 mg/ml); Rupafin (rupatadine fumarate, antiallergic, at 2.56 mg/ml); Selozok (metoprolol succinate, antihypertensive, at 10.0 mg/ml); Sinvalip (simvastatin, hypolipidemic, at 4.0 mg/ml); Somalgin cardio (acetylsalicylic acid, analgesic and antipyretic, at 20.0 mg/ml); Spasfon LYOC (phloroglucinol, antispasmodic, at 16.0 mg/ml); Tamisa 30 (gestodene and ethinyl estradiol, contraceptive, at 15.0 and 0.006 mcg/ml); Toragesic (kerotolac trometamol, analgesic, at 2.0 mg/ml); Transamin (tranexamic acid, antihemorrhagic, at 50.0 mg/ml); Tylenol (paracetamol and pseudoephedrine chloridrate, analgesic and antipyretic, at 100.0 and 6.0 mg/ml); Tylex (paracetamol and codeine fosfate, analgesic and antipyretic, at 100.0 and 6.0 mg/ml); Vasativ (cilostazol, antiplatelet**,** at 20.0 mg/ml); Vertix (flunarizine dihydrochloride, calcium channel blocker, at 2.0 mg/ml).

Discussion

The *in vitro* study showed that *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 can survive against the stress conditions found in the gastrointestinal tract. However, the *in vitro* trials involving pH, bile salt, and NaCl tolerance, it cannot predict patterns of behavior in the human body because most of methodologies used to analyses the potential probiotics strains to stressful conditions are static models, which cannot foresee the gradual changes of pH values and bile salts in the GIT (Todorov et al. 2011). To be considered a probiotic culture, some criteria should be fulfilled, including the ability to tolerate and survive the acidic environment of the stomach and the bile salts presented into the small intestine. *Lc. mesenteroides* subsp. *mesenteroides* isolated from fish intestine survived better under neutral conditions, which is similar to the neutral condition present in the intestine (Allameh et al. 2012). Previous studies also reported that LAB were able to grow and survive at low pH levels (Mishra and Prasad 2005; Duangjitcharoen et al. 2008; Allameh et al. 2012; Divya et al. 2012). However, the tolerance of gastric transit has been found to be variable among the strains (Vinderola and Reinheimer 2003).

The physiological concentration of human bile varies and depends on race, physiological conditions, and gender. Studies have shown that all probiotic strains should be able to grow and survive in the presence of up to 0.3 % bile salts (Divya et al. 2012). *Lc. mesenteroides* YML003 presented high survival rates in 5 % bile (Seo et al. 2012). According to Ouwehand et al. (1999, Aswathy et al. (2008), Shobharani and Agrawal (2011), Meira et al. (2012), LAB isolated from different sources were relatively tolerant to bile salts. *Lactobacillus acidophilus* NIT survived and grew in the presence of 1.0-3.0 % bile salts (Pan et al. 2009). In addition, Allameh et al. (2012) and Todorov et al. (2008) reported that bile salt affected the growth rate of *Lc. mesenteroides* subsp. *mesenteroides* and limited its viability.

Many attempts have been made to demonstrate the reduction of cholesterol concentrations in human blood through bile salt hydrolases from LAB strains, but it has not yet been proved (Kumar et al. 2013). Deconjugation of bile salts by LAB increases the demand for

cholesterol which, in turn, prompts the synthesis of more bile salts in the liver. This process may lead to a reduction in serum cholesterol (Kumar et al. 2013). Different species of LAB have also presented the ability to deconjugate bile salts (Vinderola and Reinheimer 2003; Kumar et al. 2013). Furthermore, to the best of our knowledge, there are currently no published studies on the ability of *Lc. mesenteroides* subsp. *mesenteroides* to deconjugate bile salts.

Most LAB are halotolerant, which is an important characteristic for their use in dairy products, especially cheeses, and *Leuconostoc* can growth in the presence of 7.0 % NaCl (Hemme and Foucaud-Scheunemann 2004). It is important to consider that *Lc. mesenteroides* subsp. *mesenteroides* normally has a very limited growth at 5 ^{o}C and in the presence of NaCl, the best condition observed in our study was at 30 ^{o}C, probably because this is the optimum growth temperature for this microorganism.

The aggregation phenotype can help probiotic cultures to adhere, colonize the GIT and modulate the immune system (Ouwehand and Vesterlund 2004). The strains evaluated in the present study were found to have higher auto-aggregation values than the indicator microorganisms. This result suggests the specific affinity of *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 to the GIT, probably due the formation of biofilms, which can helps the colonization of SJRP55 strain to the epithelial intestine cells. Similar results were reported by Ouwehand et al. (1999), Collado et al. (2007) and Xu et al. (2009) with different species of potential probiotic LAB. Co-aggregation has been related to the ability of potential probiotic strain to interact closely with pathogens (Collado et al. 2007). This characteristic can increase the competition of receptor epithelial intestine cells and may decrease the presence of undesired microorganism in the intestine due the production of antimicrobial compounds or other factors. The results of co-aggregation observed here were strain-specific and incubation temperature-dependent. A downside is that high co-aggregation values may be a potential virulence factor, which genetical material can be transferred by conjugation (Hemme and Foucaud-Scheunemann 2004; Ouwehand and Vesterlund 2004).

Hydrophobicity is one of the physicochemical properties that can facilitate the first contact between the microorganisms and the host cells (Shobharani and Agrawal 2011). These

results should be interpreted with caution because the adherence feature to intestine does not necessarily mean an *in vivo* adhesion would occur (Bautista-Gallego et al. 2013). Moreover, cell surface hydrophobicity is strain-specific and the presence of different nutrients or carrier food matrices may influence the expression of adhesion genes in the microorganisms (Ouwehand and Vesterlund 2004; Raghavendra and Halami 2009). The results obtained by the hydrophobicity tests for *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 were higher than some other strains reported in the literature (Aswathy et al. 2008; Raghavendra and Halami 2009): LAB isolated from fermented vegetables, sourdough, milk products, and sheep and human excreta (23.0 to 73.0 %) and *Pediococcus pentosaceus* CFR R38 and CFR R35, and *Lactobacillus rhamnosus* GG ATCC 53510 isolated from different sources (44.8 to 59.0 %). Results also differed in the case of *Lc. paramesenteroides* isolated from cheddar cheese (46.11 %) (Shobharani and Agrawal 2011). Moreover, different compounds commonly used to evaluate the hydrophobicity (*n*-hexadecane, xylene and toluene) can lead to different results. In addition, the hydrophobicity property can be related to the auto-aggregation and co-aggregation of strains.

The ability to adhere to the Caco-2 cells seems to be strain-dependent. *Leuconostoc* spp. is not a common species colonizer genus in the intestinal tract (Hemme and Foucaud-Scheunemann 2004). However, studies suggest that the intake of live and also heat-killed *Lc. mesenteroides* 1RM3 might prevent *Listeria monocytogenes* entero-gastric administrated from invading Caco-2 cells and infecting A/J mice (Nakamura et al. 2012). This adherence characteristic is also present in other isolated LAB (Perea Velez et al. 2007; Deepika et al. 2011; Argyri et al. 2013). The ability to adhere to mucosal surfaces in the intestine plays an important role in defining a probiotic culture. The colonization of the intestine by probiotic strains can generate beneficial biological responses: they can influence immune system and increase the competition with pathogens in the intestine (Adams 2010). The *in vitro* adhesive proprieties of *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 can indicate that the strain is able to adhere to the intestine of the host and may be able to activate the genes that encode antimicrobial compounds, such as bacteriocins, which can act against pathogens present in the GIT.

Moreover, previous studies showed that *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 possesses anti-*Listeria* activity (Paula et al. 2012).

Some consumers of dairy products are lactose-intolerant, a condition which causes discomfort after the digestion of milk (Raghavendra and Halami 2009). *β*–galactosidase hydrolyses lactose to galactose and glucose, which aids lactose digestion in the intestine. When people have limited digestion or are lactose intolerant, the consumption of dairy products causes discomfort, gases and flatulence. Since LAB cultures can produce this enzyme, it is becoming important for the dairy industry to explore this property, in order to help lactose-intolerant consumers. Previous studies with *Lactobacillus delbrueckii* subsp. *lactis* (Guglielmotti et al. 2007), *Pediococcus pentosaceus* and *Lact. rhamnosus* (Raghavendra and Halami 2009), *Lc. paramesenteroides* (Shobharani and Agrawal 2011) and *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus casei*, and *Lactobacillus parabuchneri* (Meira et al. 2012) also revealed *β*–galactosidase activity.

The knowledge of the enzymatic profile of the future probiotic strains is an important point, since some enzymes, such as β-glucuronidase may be related with toxic reactions and formation of harmful metabolites. *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 was negative for β-glucuronidase activity based on the results obtained from API-ZYM kit. However, various studies suggested that the enzymatic activity of *Lc. mesenteroides* cultures is strain-specific (Thapa et al. 2006; Rai et al. 2010; Ryu and Chang 2013).

Probiotic cultures have been exploited extensively by dairy industry as a tool for the development of novel functional products (Vasiljevic and Shah 2008). The viability of probiotics cultures is a major to concern, since it can affect the probiotic characteristics of the products. In contrast, studies have been showing that probiotic dead cells also exhibit beneficial effects to the host (Adams 2010). In our study, tests used to study the stability of *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 in fermented milk, during the storage condition, alone and in co-culture with *St. thermophilus* TA040, showed that this microorganism can survive at desirable levels during fermentation and storage period and it is a good candidate as a probiotic strain in preparation of fermented milk products. Moreover, the use of *Lc.*

mesenteroides subsp. *mesenteroides* SJRP55 as co-culture with *St. thermophilus* TA040 showed that this microorganism can be applied as an adjunct culture in fermented milk.

The production of exopolyssacharides, a common compound produced by *Leuconostoc* species can improve the viscosity, texture and mouthfeel of dairy products (Hemme and Foucaud-Scheunemann 2004; Ruas-Madiedo et al. 2005). We need to underline that in the industrial production of fermented milk products, including yoghurt, viscosity and texture are critical characteristics for consumers' acceptance of the new products on the market.

Some authors have been studying the technological characteristics of *Lc. mesenteroides* (Nieto-Arribas et al. 2010; Cardamone et al. 2011;), the presence and behavior of this microorganism in the microbial ecology of kefir grains (Hsieh et al. 2012); however up to date, the application of *Lc. mesenteroides* as both as a potential probiotic and bacteriocinogenic strain has not been reported yet.

The antibiotics that the *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 showed resistance are used for human infections and, depending on the classification, can act against Gram-positive or Gram-negative pathogenic bacteria. Antibiotic resistance is a worldwide public health problem (Ammor and Mayo 2007). The concern over this problem is in part due to the excessive and indiscriminate use of these compounds in humans, agriculture, and livestock. Moreover, LAB, pathogenic bacteria, and opportunistic bacteria can acquire and/or transmit antibiotic resistance and virulence genes by transposons and plasmids, resulting in conjugation or transformation (Grattepanche et al. 2008). Some microorganisms can be intrinsically resistant to antibiotics. In general, *Leuconostoc* sp. has an intrinsic resistance to vancomycin due to particular characteristics of its cell wall, which presents D-lactate instead of a D-alanine in the peptidoglycan (Hemme and Foucaud-Scheunemann 2004; Aswathy et al. 2008; Shobharani and Agrawal 2011). Natural (horizontal) gene transfer by *Leuconostoc* spp. can happen (Dicks et al. 2009), but it is seldom found (Hemme and Foucaud-Scheunemann 2004). However, no cases of infection by consumption of dairy products containing *Leuconostoc* spp. has been reported, which makes this microorganism recognized as safe. Moreover, LAB resistant to antibiotics can proliferate in the gut and maintain microbial balance, thereby reducing the levels of

opportunistic microorganisms. Previous studies showed that *Leuconostoc* spp. is resistant to fosfomycin, ‘‘old’’ quinolones, and glycopeptides; it is also susceptible to or leads to intermediate sensitivity to macrolides and tetracyclines (Hemme and Foucaud-Scheunemann 2004). *Lc. mesenteroides* sp. *mesenteroides* was resistant to streptomycin and intermediate to amoxicillin and kanamycin (Allameh et al. 2012), whereas *Lc. paramesenteroides* isolated from kimchi was resistant to tetracycline, gentamicin, neomycin, streptomycin and sulfisoxazole (Shobharani and Agrawal 2011). In summary, antibiotic resistance seems to be strain-dependent and related to the environment in which the strain was isolated. A subject of concern regarding LAB as potential probiotic strains is the transference of resistance genes to pathogenic bacteria present in the GIT, which may pose a risk for the host, particularly in the case of immunocompromised people, the elderly, pregnant women, and newborns (Aymerich et al. 2006; Devirgiliis et al. 2011).

Nowadays, lifestyle, stress and inadequate food intake are raising the consumption of different groups of medications for pain and other kinds of illness. However, many consumers undergoing these therapies are not aware of the side effects of these compounds. In our study, the *in vitro* tests showed that different medications can inhibit *Lc. mesenteroides* subsp. *mesenteroides* SJRP55. Analgesic and anti-inflammatory medications are commonly used by many populations, and in many countries, including Brazil, they are often available without a prescription. The negative effect of these drugs against potential probiotic LAB seems to be common, and has been observed in other studies (Todorov et al. 2007; Todorov et al. 2011; Todorov et al. 2012). Moreover, the antihypertensive medication analyzed in our study, known as Cozaar is used in long-term treatments for a certain chronic disease. When compared to other antihypertensives, the substance present in this drug seems to be responsible for the inhibition of the *Lc. mesenteroides* subsp. *mesenteroides* SJRP55.

The minimal inhibitory concentration (MIC) values (Table 2) play an important role in the proper evaluation of the effect of medications on probiotic bacteria (Todorov et al. 2012). The presence of the compound metamizole in analgesic and anti-inflammatory drugs may be the responsible for the low MIC values found in the *in vitro* test. Metamizole or dypirone is a

nonsteroidal anti-inflammatory agent that is prohibited in most industrialized countries because of the risk of fatal agranulocytosis, but it is widely used in Latin America, Africa and Asia. This compound binds to neutrophil membranes, creating a novel antigen that induces antibody formation. The resultant immune response causes both peripheral and bone marrow cell lysis (Bonkowsky et al. 2002; Hedenmalm and Spigset 2002; Yiğit and Soyuncu 2012). These results are cause for concern, because the intake of these drugs in Brazil is very frequent at all ages for different therapies. The medications known commercially in Brazil as Dorflex and Neosaldina did not affect the growth of *Lactobacillus casei* Shirota or *Lactobacillus casei* LC01 (De Carvalho et al. 2009). Moreover, the *in vivo* studies should be done to better understand the interaction between medication and probiotic potential cultures.

This is the first study with *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 isolated from water buffalo mozzarella cheese that showed desirable probiotic characteristics using *in vitro* testing and viability in fermented milk under storage conditions. *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 may be a good candidate for further *in vivo* investigation studies. These studies will allow us to better understand the strain's potential health benefits, as well as its possible industrial applications for the development of functional foods.

Acknowledgments

The authors are grateful to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Brazil, Project N° 2010/09302-1), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil) for the financial support and to Dr. Bruno Moreira Carneiro from Sao Paulo State University for support with Caco-2 cells study.

Conflict of interest

The authors declare that there is no conflict of interest.

References

Adams CA (2010) The probiotic paradox: live and dead cells are biological response modifiers. Nutr Res Rev 23 (1):37-46. doi:10.1017/S0954422410000090

Agarwal KN, Bhasin SK (2002) Feasibility studies to control acute diarrhoea in children by feeding fermented milk preparations Actimel and Indian Dahi. Eur J Clin Nutr 56 Suppl 4:S56-59. doi:10.1038/sj.ejcn.1601664

Allameh SK, Daud H, Yusoff FM, Saad CR, Ideris A (2012) Isolation, identification and characterization of *Leuconostoc mesenteroides* as a new probiotic from intestine of snakehead fish (*Channa striatus*). Afr J Biotechnol 11 (16). doi:10.5897/ajb11.1871

Amara AA, Shibl A (2013) Role of probiotics in health improvement, infection control and disease treatment and management. Saud Pharm J. doi:10.1016/j.jsps.2013.07.001

Ammor MS, Mayo B (2007) Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: An update. Meat Sci 76 (1):138-146. doi:10.1016/j.meatsci.2006.10.022

Argyri AA, Zoumpopoulou G, Karatzas KA, Tsakalidou E, Nychas GJ, Panagou EZ, Tassou CC (2013) Selection of potential probiotic lactic acid bacteria from fermented olives by *in vitro* tests. Food Microbiol 33 (2):282-291. doi:10.1016/j.fm.2012.10.005

Aswathy RG, Ismail B, John RP, Nampoothiri KM (2008) Evaluation of the probiotic characteristics of newly isolated lactic acid bacteria. Appl Biochem Biotech 151 (2-3):244-255. doi:10.1007/s12010-008-8183-6

Aymerich T, Martin B, Garriga M, Vidal-Carou MC, Bover-Cid S, Hugas M (2006) Safety properties and molecular strain typing of lactic acid bacteria from slightly fermented sausages. J Appl Microbiol 100 (1):40-49. doi:10.1111/j.1365-2672.2005.02772.x

Bautista-Gallego J, Arroyo-López FN, Rantsiou K, Jiménez-Díaz R, Garrido-Fernández A, Cocolin L (2013) Screening of lactic acid bacteria isolated from fermented table olives with probiotic potential. Food Res Int 50 (1):135-142. doi:10.1016/j.foodres.2012.10.004

Bonkowsky JL, Frazer JK, Buchi KF, Byington CL (2002) Metamizole use by latino immigrants: A common and potentially harmful home remedy. Pediatr 109 (6):1-3. doi:10.1542/peds.109.6.e98

Cardamone L, Quiberoni A, Mercanti DJ, Fornasari ME, Reinheimer JA, Guglielmotti DM (2011) Adventitious dairy *Leuconostoc* strains with interesting technological and biological properties useful for adjunct starters. Dairy Sci Technol 91 (4):457-470. doi:10.1007/s13594-011-0022-9

Collado MC, Surono I, Meriluoto J, Salminen S (2007) Indigenous dadih lactic acid bacteria: cell-surface properties and interactions with pathogens. J Food Sci 72 (3):M89-93. doi:10.1111/j.1750-3841.2007.00294.x

Dave RI, Shah NP (1996) Evaluation of media for selective enumeration of *Streptococcus thermophillus, Lactobacillus delbrueckii* ssp. *bulgaricus, Lactobacillus acidophilus,* and Bifidobacteria. J Dairy Sci 79:1529–1536

de Azevedo MS, Innocentin S, Dorella FA, Rocha CS, Mariat D, Pontes DS, Miyoshi A, Azevedo V, Langella P, Chatel JM (2013) Immunotherapy of allergic diseases using

probiotics or recombinant probiotics. J Appl Microbiol 115 (2):319-333. doi:10.1111/jam.12174

De Carvalho KG, Kruger MF, Furtado DN, Todorov SD, M. FBDG (2009) Evaluation of the role of environmental factors in the human gastrointestinal tract on the behavior of probiotic cultures of *Lactobacillus casei* Shirota and *Lactobacillus casei* LC01 by the use of a semi-dynamic in vitro model. Ann Microbiol 59 (3):439-445

Deepika G, Rastall RA, Charalampopoulos D (2011) Effect of food models and low-temperature storage on the adhesion of *Lactobacillus rhamnosus* GG to Caco-2 cells. J Agr Food Chem 59 (16):8661-8666. doi:10.1021/jf2018287

Devirgiliis C, Barile S, Perozzi G (2011) Antibiotic resistance determinants in the interplay between food and gut microbiota. Gene Nutr 6 (3):275-284. doi:10.1007/s12263-011-0226-x

Dicks LMT, Todorov SD, Franco BDGM (2009) Current status of antibiotic resistance in lactic acid bacteria In: Bonilla AR, Muniz KP (eds) Antibiotic resistance: causes and risk factors. Nova Publisher, New York, pp 1-46

Divya JB, Varsha KK, Nampoothiri KM (2012) Newly isolated lactic acid bacteria with probiotic features for potential application in food industry. Appl Bioch Biotech 167 (5):1314-1324. doi:10.1007/s12010-012-9561-7

Doyle RJ, Rosenberg M (1995) Measurement of microbial adhesion to hydrophobic substrates. Method Enzymol 253:542-550

Duangjitcharoen Y, Kantachote D, Ongsakul M, Poosaran N, Chaiyasut C (2008) Selection of probiotic lactic acid bacteria isolated from fermented plant beverages. Pakistan J Biol Sci: PJBS 11 (4):652-655

FAO/WHO (2002) Guidelines for the evaluation of probiotics in food. Report of a joint Food and Agriculture Organization (FAO) of the United Nations/World Health Organization (WHO) working group on drafting guidelines for the evaluation for the probiotics in food.

Fontana L, Bermudez-Brito M, Plaza-Diaz J, Muñoz-Quezada S, Gil A (2013) Sources, isolation, characterization and evaluation of probiotics. Brit J Nutr 109:S35-50

Grattepanche F, Miescher-Schwenninger S, Meile L, Lacroix C (2008) Recent developments in cheese cultures with protective and probiotic functionalities. Dairy Sci Technol 88 (4-5):421-444. doi:10.1051/dst:2008013

Guglielmotti DM, Marcó MB, Golowczyc M, Reinheimer JA, Quiberoni AdL (2007) Probiotic potential of *Lactobacillus delbrueckii* strains and their phage resistant mutants. . Int Dairy J 17 (8):916-925. doi:10.1016/j.idairyj.2006.11.004

Hedenmalm K, Spigset O (2002) Agranulocytosis and other blood dyscrasias associated with dipyrone (metamizole). Eur J Clin Pharmacol 58 (4):265-274. doi:10.1007/s00228-002-0465-2

Hemme D, Foucaud-Scheunemann C (2004) *Leuconostoc*, characteristics, use in dairy technology and prospects in functional foods. Int Dairy J 14 (6):467-494. doi:10.1016/j.idairyj.2003.10.005

Hsieh HH, Wang SY, Chen TL, Huang YL, Chen MJ (2012) Effects of cow's and goat's milk as fermentation media on the microbial ecology of sugary kefir grains. Int J Food Microbiol 157 (1):73-81. doi:10.1016/j.ijfoodmicro.2012.04.014

Kos B, Šušković J, Beganović J, Gjuračić K, Frece J, Iannaccone C, Canganella F (2007) Characterization of the three selected probiotic strains for the application in food industry. World J Microbiol and Biotechnol 24 (5):699-607. doi:10.1007/s11274-007-9528-y

Kumar A, Kumar M, Ghosh M, Ganguli A (2013) Modeling in vitro cholesterol reduction in relation to growth of probiotic *Lactobacillus casei*. Microbiol Immunol 57 (2):100-110. doi:10.1111/1348-0421.12008

Lee MR, Huang YT, Lee PI, Liao CH, Lai CC, Lee LN, Hsueh PR (2011) Healthcare-associated bacteraemia caused by *Leuconostoc* species at a university hospital in Taiwan between 1995 and 2008. J Hosp Infec 78 (1):45-49. doi:10.1016/j.jhin.2010.11.014

Meira SM, Helfer VE, Velho RV, Lopes FC, Brandelli A (2012) Probiotic potential of *Lactobacillus* spp. isolated from Brazilian regional ovine cheese. J Dairy Res 79 (1):119-127. doi:10.1017/S0022029911000884

Mishra V, Prasad DN (2005) Application of *in vitro* methods for selection of *Lactobacillus casei* strains as potential probiotics. Int J Food Microbiol 103 (1):109-115. doi:10.1016/j.ijfoodmicro.2004.10.047

Nakamura S, Kuda T, An C, Kanno T, Takahashi H, Kimura B (2012) Inhibitory effects of *Leuconostoc mesenteroides* 1RM3 isolated from narezushi, a fermented fish with rice, on *Listeria monocytogenes* infection to Caco-2 cells and A/J mice. Anaerobe 18 (1):19-24. doi:10.1016/j.anaerobe.2011.11.006

Nieto-Arribas P, Sesena S, Poveda JM, Palop L, Cabezas L (2010) Genotypic and technological characterization of *Leuconostoc* isolates to be used as adjunct starters in Manchego cheese manufacture. Food Microbiol 27 (1):85-93. doi:10.1016/j.fm.2009.08.006

Ouwehand AC, Kirjavainen PV, Grönlund M-M, Isolauri E, Salminen SJ (1999) Adhesion of probiotic micro-organisms to intestinal mucus. Int Dairy J 9 (9):623-630

Ouwehand AC, Vesterlund S (2004) Antimicrobial components from lactic acid bacteria. In: Salminen S, von Wright A, Ouwehand A (eds) Lactic acid bacteria microbiological and functional aspects 3edn. Marcel Dekker, New York, pp 375-396

Pan X, Chen F, Wu T, Tang H, Zhao Z (2009) The acid, bile tolerance and antimicrobial property of *Lactobacillus acidophilus* NIT. Food Control 20 (6):598-502. doi:10.1016/j.foodcont.2008.08.019

Paula AT, Jeronymo ABO, Silva LF, Todorov SD, Franco BDGM, Penna ALB *Leuconostoc mesenteroides* SJRP55: bacteriocionogenic strain isolated from water buffalo mozzarella cheese. In: 3rd International Symposium on Antimicrobial Peptides: Today knowledge and future applications, Lille, 2012.

Perea Velez M, Hermans K, Verhoeven TL, Lebeer SE, Vanderleyden J, De Keersmaecker SC (2007) Identification and characterization of starter lactic acid bacteria and probiotics from Columbian dairy products. J Applied Microbiol 103 (3):666-674. doi:10.1111/j.1365-2672.2007.03294.x

Raghavendra P, Halami PM (2009) Screening, selection and characterization of phytic acid degrading lactic acid bacteria from chicken intestine. Int J Food Microbiol 133 (1-2):129-134. doi:10.1016/j.ijfoodmicro.2009.05.006

Rai AK, Tamang JP, Palni U (2010) Microbiological studies of ethnic meat products of the Eastern Himalayas. Meat Sci 85 (3):560-507. doi:10.1016/j.meatsci.2010.03.006

Rayes N, Seehofer D, Theruvath T, Schiller RA, Langrehr JM, Jonas S, Bengmark S, Neuhaus P (2005) Supply of pre- and probiotics reduces bacterial infection rates after liver transplantation--a randomized, double-blind trial. Am J Transplant 5 (1):125-130. doi:10.1111/j.1600-6143.2004.00649.x

Rayes N, Seehofer D, Neuhaus P (2009) Prebiotics, probiotics, synbiotics in surgery--are they only trendy, truly effective or even dangerous? Langenbecks Arch Surg/Deutsche Gesellschaft fur Chirurgie 394 (3):547-555. doi:10.1007/s00423-008-0445-9

Reis JA, Casarotti SN, Paula AT, Penna ALB (2011) Probióticos e seus efeitos terapêuticos na saúde humana. Cultura Acadêmica, São José do Rio Preto

Ruas-Madiedo P, Alting AC, Zoon P (2005) Effect of exopolysaccharides and proteolytic activity of *Lactococcus lactis* subsp. *cremoris* strains on the viscosity and structure of fermented milks. Int Dairy J 15 (2):155-164. doi:10.1016/j.idairyj.2004.05.009

Ryu EH, Chang HC (2013) *In vitro* study of potentially probiotic lactic acid bacteria strains isolated from kimchi. Ann Microbiol 63 (4):1387-1395. doi:10.1007/s13213-013-0599-8

Seo BJ, Rather IA, Kumar VJ, Choi UH, Moon MR, Lim JH, Park YH (2012) Evaluation of *Leuconostoc mesenteroides* YML003 as a probiotic against low-pathogenic avian influenza (H9N2) virus in chickens. J Appl Microbiol 113 (1):163-171. doi:10.1111/j.1365-2672.2012.05326.x

Shiby VK, Mishra HN (2013) Fermented milks and milk products as functional foods--a review. Crit Rev Food Sci Nutr 53 (5):482-496. doi:10.1080/10408398.2010.547398

Shobharani P, Agrawal R (2011) A potent probiotic strain from cheddar cheese. Indian J Microbiol 51 (3):251-258. doi:10.1007/s12088-011-0072-y

Silva LF (2010) Identificação e caracterização da microbiota lática isolada de queijo mussarela de búfala. Universidade Estadual Paulista, São José do Rio Preto

Singh K, Kallali B, Kumar A, Thaker V (2011) Probiotics: A review. Asian Pac J Trop Biomed 1 (2):S287-290. doi:10.1016/s2221-1691(11)60174-3

Tamang JP, Tamang B, Schillinger U, Guigas C, Holzapfel WH (2009) Functional properties of lactic acid bacteria isolated from ethnic fermented vegetables of the Himalayas. International Int J Food Microbiol 135 (1):28-33. doi:10.1016/j.ijfoodmicro.2009.07.016

Thapa N, Pal J, Tamang JP (2006) Phenotypic identification and technological properties of lactic acid bacteria isolated from traditionally processed fish products of the Eastern Himalayas. Int J Food Microbiol 107 (1):33-38. doi:10.1016/j.ijfoodmicro.2005.08.009

Todorov SD, Botes M, Danova ST, Dicks LM (2007) Probiotic properties of *Lactococcus lactis* ssp. *lactis* HV219, isolated from human vaginal secretions. J Appl Microbiol 103 (3):629-639. doi:10.1111/j.1365-2672.2007.03290.x

Todorov SD, Botes M, Guigas C, Schillinger U, Wiid I, Wachsman MB, Holzapfel WH, Dicks LM (2008) Boza, a natural source of probiotic lactic acid bacteria. J Appl Microbiol 104 (2):465-477. doi:10.1111/j.1365-2672.2007.03558.x

Todorov SD, Furtado DN, Saad SM, Tome E, Franco BD (2011) Potential beneficial properties of bacteriocin-producing lactic acid bacteria isolated from smoked salmon. J Appl Microbiol. doi:10.1111/j.1365-2672.2011.04950.x

Todorov SD, Leblanc JG, Franco BD (2012) Evaluation of the probiotic potential and effect of encapsulation on survival for *Lactobacillus plantarum* ST16Pa isolated from papaya. World J Microbiol Biotechnol 28 (3):973-984. doi:10.1007/s11274-011-0895-z

Vasiljevic T, Shah NP (2008) Probiotics—From Metchnikoff to bioactives. Int Dairy J 18 (7):714-728. doi:10.1016/j.idairyj.2008.03.004

Vinderola CG, Reinheimer JA (2003) Lactic acid starter and probiotic bacteria: a comparative "in vitro" study of probiotic characteristics and biological barrier resistance. Food Res Int 36 (9-10):895-804. doi:10.1016/s0963-9969(03)00098-x

Xu H, Jeong HS, Lee HY, Ahn J (2009) Assessment of cell surface properties and adhesion potential of selected probiotic strains. Lett Appl Microbiol 49 (4):434-442. doi:10.1111/j.1472-765X.2009.02684.x

Yiğit Ö, Soyuncu S (2012) Dipyrone, great killer or innocent as any drug: Two case reports presenting rare and life-threatening adverse effects of dipyrone. Turkiye Klinikleri J Med Sci 32 (3):832-836. doi:10.5336/medsci.2010-18328

Capítulo III

Leuconostoc mesenteroides SJRP55: a bacteriocinogenic strain isolated from Brazilian water buffalo mozzarella cheese

Este Capítulo será submetido à Revista *Probiotics and Antimicrobial Proteins*

Leuconostoc mesenteroides SJRP55: a bacteriocinogenic strain isolated from Brazilian water buffalo mozzarella cheese

Aline T. de Paula, Ana Beatriz Jeronymo-Ceneviva, Luana F. Silva, Svetoslav D. Todorov, Bernadette D. G. M. Franco, Yvan Choiset, Thomas Haertlé, Jean-Marc Chobert, Xavier Dousset, Ana Lúcia B. Penna

Aline de Paula · Ana Beatriz Jeronymo-Ceneviva · Luana Silva · Ana Lúcia Penna (✉)

UNESP - Sao Paulo State University, Food Engineering and Technology Department, Rua Cristovão Colombo, 2265, 15.054-000, +55 17 3221-2266, São José do Rio Preto, Brazil. E-mail: analucia@ibilce.unesp.br

Svestoslav Todorov · Bernadette Franco

USP - Sao Paulo University, Faculty of Pharmaceutical Sciences, Department of Food Science and Experimental Nutrition, Av. Prof. Lineu Prestes, 580, bl. 13B, 05508-000, Sao Paulo, Brazil

Yvan Choiset · Thomas Haertlé · Jean-Marc Chobert

UR 1268, INRA Biopolymères Interactions Assemblages Equipe FIP, B.P. 71627, 44316 Nantes Cedex 03, France

Xavier Dousset

LUNAM Université, Oniris, UMR1014 Secalim Rue Géraudière, B.P. 82225, 44322 Nantes Cedex 03, France 5 INRA Nantes F-44307-France

Abstract

The production of bacteriocins by *Leuconostoc mesenteroides* represents an important opportunity for exploration of their potential use for industrial purpose. The antimicrobial proteinaceous compounds produced by *Leuc. mesenteroides* subsp. *mesenteroides* SJRP55 strain were characterized and purified. Cell-free supernatant of SJRP55 *Leuc. mesenteroides subsp. mesenteroides SJRP55* produced antibacterial compounds of *Listeria spp.* strains and no inhibition against *Lactobacillus* spp. The antimicrobial substances were resistant to high temperatures, low pH values, presence of chemical compounds, but sensitive to proteolytic enzymes and resistant to α-amylase, lipase and catalase enzymes. The optimal temperature for active peptides production was 25 °C. The antimicrobial compounds showed a slight decrease on the growth of *Listeria innocua* and *Listeria monocytogenes* strains and reduced one log cycle of viable cells of *Leuc. mesenteroides.* No adsorption to the producer cells was found. The antimicrobial compounds were purified by ammonium sulphate precipitation, affinity column and reverse-phase chromatography. Mass spectrometry and aminoacids analyses showed that the bacteriocins produced by *Leuc. mesenteroides* subsp. *mesenteroides SJRP55* were identical to mesentericin Y105 and B105. The strain presented positive results for some genes encoding virulence factors, such as enterococcal surface protein (*esp*), collagen adhesion (*ace*) and intrinsic vancomycin resistance (*vanA*)*;* however, biogenic amines encoding genes were not observed. The results showed the in vitro probiotic potential of *Leuc. mesenteroides* subsp. *mesenteroides* SJRP55 suggested and its application as a promisor biopreservative culture in fermented milk. The purified bacteriocins can also be applied as a hurdle technology in food preservation.

Key words: mesentericin Y105 and mesentericin B105, *Leuconostoc mesenteroides* subsp. *mesenteroides*, antimicrobial activity, purification, lactic acid bacteria.

1. Introduction

Lactic acid bacteria (LAB) are found in dairy products, beverages, meat, vegetables and as natural microbiota of human, plants, and animals. The exploration and understanding of these microorganisms play an important role for food industry. LAB can improve the sensorial and nutritional properties of fermented products and additionally, they can produce metabolic compounds, such as bacteriocins, which can be used as natural food preservatives, extending the shelf-life of many food products [1, 2].

Bacteriocins are an abundant and diverse group of ribosomally synthesized antimicrobial peptides produced by *Bacteria* and *Archaea* [3]. The production of bacteriocins by LAB has attracted the attention of dairy industry, which is interesting to decrease the use of chemical additives in their products to meet consumers' expectations for healthier foods. The spectrum of activity of bacteriocins against certain foodborne pathogens and spoilage microorganisms, the resistance to high temperatures, and low pH together with facility to be digested by human proteolytic enzymes make bacteriocins an interesting tool for the application in food preservation [4, 5]. However, the discovery and application of new bacteriocins produced by LAB is still a challenge.

Leuconostoc spp. are heterofermentative LAB found mainly in vegetables, cereal, silage, fruits, wine, fish, dairy products and meat. Some strains isolated from different food matrices are capable to produce bacteriocins [4, 6-10, 11-15]. According to their biochemical properties most of these bacteriocins are classified according to Klaenhammer [16] in class II, which are small (<10 kDa), heat-stable, do not contain lanthionine, membrane active peptides, with a consensus sequence (YGNGVXCaaCVaaV) in the N-terminus and anti-*Listeria* activity [17].

The capacity of *L. monocytogenes* to survive in different extreme environmental conditions makes this microorganism difficult to control in food system. It is a subject of concern, since traditional and artisanal fermented foods, such as some cheeses, are produced

without the use of starter cultures, using only autochthonous microbiota, which leads to an increase in the risk of contamination by *L. monocytogenes* [17].

Technologically *Leuconostoc* strains can present interesting characteristics, such as production of dextran, acetaldehyde, diacetyl and acetoin, lipolytic and proteolytic enzymes, low amount of acid and ability to grow under stress conditions (acid, high salt content and elevated temperature) [18]. Otherwise, depending on the application of this microorganism, a considerable amount of carbon dioxide can be produced during fermentation of carbohydrates [19]. In addition, the bacteriocins, in combination with different physicochemical hurdles, can be used for food biopreservative, such as in food to control the growth of *L. monocytogenes* [15, 20]. The aim of this study was to characterize and purify the bacteriocins produced by *Leuc. mesenteroides* subsp. *mesenteroides* SJRP55 isolated from Brazilian water buffalo mozzarella cheese.

2. Materials and methods

2.1. Culture media and incubation conditions

Leuc. mesenteroides subsp. *mesenteroides* SJRP55 was isolated from water buffalo mozzarella cheese and identified by whole 16S rDNA gene sequencing [21]. The strain was grown aerobically at 30 °C in de Man-Rogosa-Sharpe (MRS) broth (Difco Laboratories, Detroit, MI, USA) for 18–24 h and stored at –80 °C in the presence of 20 % of glycerol (v/v). In addition, the bacterial strains used as indicators were cultured in the conditions listed in Table 3.

2.2. Determination of the antimicrobial compounds

Agar-spot assay described by Schillinger and Lücke [22] was used to evaluate the antimicrobial spectrum against indicator microorganisms. Cell-free supernatant (CFS) was obtained from an 18 h-old culture of *Leuc. mesenteroides* subsp. *mesenteroides* SJRP55 (1 % inoculum, v/v, at 25

°C) grown in 200 ml of MRS broth, followed by centrifugation (12.500 x *g*, 15 min, 4 °C). CFS pH was adjusted to 6.5 with 1 N NaOH and heated at 100 °C for 10 min. The supernatant was then sterilized through a microbiological filter (0.22 μm, TPP, Trasadingen, Switzerland). Ten microliters were spotted on the surface of MRS agar or BHI agar added of 1 % (v/v) with the indicator strains (Table 3) and incubated at 30 °C for 18 h. The formation of an inhibition zone around the spots indicated the antimicrobial activity.

2.3. Effect of enzymes, pH, temperature and chemical agents on antimicrobial compounds

Two milliliters of 100 ml (v/v) of CFS were incubated at 30 °C for 2 h in the presence of pepsin, α-chymotrypsin of bovine pancreas type II, protease of *Aspergillus saitoi*, trypsin of porcine pancreas, lipase of *Aspergillus niger*, catalase of bovine liver, and α-amylase of *Aspergillus oryzae* (all from Sigma-Aldrich, St Louis, MO, USA), at a final concentration of 1 mg/ml, then heated at 95–97 °C for 5 min to stop the enzymatic reaction.

In a parallel experiment 10 ml of CFS, with pH adjusted to 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 with 1 N HCl or 1 N NaOH were incubated at 30 °C for 2 h. After this period, the pH of the samples was readjusted to pH 6.5, before testing the antimicrobial activity.

The effect of temperature on the antimicrobial substances were also tested by heating the CFS at 30, 37, 45, 60 and 100 °C for 120 min, and at 121 °C for 20 min.

CFS was tested in presence of chemical agents such as 1 % of Tween 20, Tween 80, SDS, Triton X-100, Na-EDTA, NaCl and 1 M of urea (all from Synth, Diadema, São Paulo, Brazil) [23]. The samples were incubated at 30 °C for 2 h in the presence of the select chemicals, previous to the antimicrobial activity tests.

The antimicrobial compounds were measured by using the agar-spot test method for all tests according to Schillinger and Lücke [22]. *Leuc. mesenteroides* subsp. *mesenteroides* UCV10CET, *Listeria innocua* ATCC 33090 and *L. monocytogenes* ATCC 7644 were used as indicator microorganisms. The same procedure was conducted with partially purified (Sep-Pak C_2 column, Waters Millipore, Milford, MA, USA) bacteriocin SJRP55.

2.4. Kinetics of growth and antimicrobial compounds production

Two percent (v/v) of 18 h-old culture of *Leuc. mesenteroides* subsp. *mesenteroides* SJRP55 were inoculated in 100 ml of MRS broth and incubated at 25 °C and 30 °C. The optical density (OD_{600nm}), pH and antimicrobial activity (AU/ml) of bacteriocin were recorded every hour for 48 h. *L. monocytogenes* ATCC 7644 was used as an indicator microorganism [24]. Arbitrary unit (AU) was defined as the reciprocal of the highest dilution (2^n) that resulted in inhibition of the indicator spots [25].

2.5. Mode of action

The effect of the antimicrobial peptides on the growth of indicator microorganims was evaluated according to Todorov et al. [24]. After 3 h of incubation at 30 °C of the indicator microorganisms, 10 % (v/v) of CFS of *Leuc. mesenteroides* subsp. *mesenteroides* SJRP55 were added to 100 ml of MRS broth with *Leuc. mesenteroides* subsp. *mesenteroides* UCV10CET and to 100 ml of BHI broth with *L. innocua* ATCC 33090 and *L. monocytogenes* ATCC 7644, separately. Optical density (OD_{600nm}) was measured every hour for 12 h.

2.6. Determination of the reduction of viable cells of the indicator microorganisms in the presence of antimicrobial compounds

The cells present in an 18 h-old culture of the indicator microorganisms (*Leuc. mesenteroides* subsp. *mesenteroides* UCV10CET, *L. innocua* ATCC 33090 and *L. monocytogenes* ATCC 7644 [10^8 – 10^9 CFU/ml]) were harvested (5.000 x *g*, 5 min, 4 °C), washed twice with sterile saline solution (0.85 % NaCl) and re-suspended in 10 ml of saline solution. Five milliliters of indicator culture's suspension and 5 ml of CFS were mixed. Numbers of viable cells were determined at time zero and after 1 h at 30 °C by pour-plating on MRS agar or BHI supplemented with 1.5 %

agar. Cell suspension of indicator microorganisms without addition of CFS served as control [24].

2.7. Adsorption of the active peptides to the producer cells

An 18 h-old culture of *Leuc. mesenteroides* subsp. *mesenteroides* SJRP55 was adjusted to pH 6.0 with sterile 1 N NaOH. Cells were harvested (10.000 x *g*, 15 min, 4 °C) and washed with 10 ml of sterile 0.1 M phosphate buffer (pH 6.5). The cells were re-suspended in 10 ml of sterile 100 mM NaCl (pH 2.0 solution), stirred (100 rpm, 1 h, 4 °C), and then harvested (10.000 x *g*, 15 min, 4 °C) [26]. CFS was neutralized at pH 7.0 with sterile 1 N NaOH and the antimicrobial activity against *Leuc. mesenteroides* subsp. *mesenteroides* UCV10CET, *L. innocua* ATCC 33090 and *L. monocytogenes* ATCC was performed using the agar-spot test.

2.8. Purification of the active compounds

Bacteriocin SJRP55 was purified according to Héchard et al. [10] with modifications. *Leuc. mesenteroides* subsp. *mesenteroides* SJRP55 was grown in 1 L of MRS broth at 25 °C for 24 h, and then the cells were harvested (12.500 x *g*, 15 min, 4 °C) and the pH of the supernatant was adjusted to 6.5 – 7.0, using 1 N NaOH.

In order to obtain the crude antimicrobial preparation, the soluble peptides present in CFS were precipitated with ammonium sulfate (40 %, 60 % and 80 %) and the solution was stirred at 4 °C for 2 h. CFS was harvested (16.000 x *g*, 30 min, 4 °C), and the precipitate was recovered in 100 ml of 0.1 M phosphate buffer (pH 6.5), filtered through 0.22 µm filters and heated at 100 °C for 10 min.

In all purification steps, the pH of the collected samples was adjusted to 6.5 – 7.0 with 1 N NaOH and the antimicrobial activity against *Leuc. mesenteroides* subsp. *mesenteroides* UCV10CET and *L. monocytogenes* ATCC 7644 was performed using the agar well diffusion assay [22].

The active fraction obtained after precipitation with ammonium sulfate was then applied on affinity column Blue Sepharose High Performance (1.6 x 2.5 cm, GE Healthcare Uppsala, Sweden), equilibrated with 20 mM acetate buffer (pH 5.0). To remove colored contaminant compounds, the column was washed with 20 mM acetate buffer (pH 5.0) and then washed with 1 M NaCl in 20 mM acetate buffer (pH 5.0). To remove the NaCl, the initial buffer was applied on the column. Finally, 6 M of urea and 1 M of NaCl were added to the column (pH 3.0) and the fractions were collected using a fraction collector (Gilson, model 203, Middleton, WI, USA).

The collected fractions with an antimicrobial activity were applied on a reversed phase Sep-Pak C_2 cartridge (Waters), equilibrated with 0.1 % trifluoroacetic acid (TFA) and 80 % acetonitrile. Elution was performed using 80 % acetonitrile, 20 % isopropanol and 0.03 % TFA (v/v/v). The fractions were concentrated using a Speed-Vac concentrator (SC110A, Savant, Illkirch, France).

The active fractions were further purified by reversed phase-high performance liquid chromatography (RP-HPLC), using a Waters Alliance with Millennium software. One hundred microliters of concentrated bacteriocin was injected into an analytical RP Nucleosil C_8 (Symmetry 300 Å C_8, 5 µm, 7.8 x 100 mm, Waters) equilibrated with solvent A (0.5 % solvent B, 95 % ultra-purified water, 0.03 % TFA, [v/v/v]). Elution was performed at a flow-rate of 0.5 ml/min with a linear gradient from 0 % to 60 % of solvent B (80 % acetonitrile, 20 % isopropanol and 0.05 % TFA) for 30 min. The eluted peaks were detected by spectrophotometry, measuring the absorbance between 220 and 280 nm with a photodiode array detector (PDA 996; Waters), and collected manually. The active fractions were concentrated using a Speed-Vac concentrator, and recovered and re-suspended in 50 µl phosphate buffer (0.2 M, pH 6.5). The concentrated purified bacteriocin was re-injected on the column using the same conditions, in order to improve the purification.

Total protein concentration for each sample in different purification steps was determined using the bicinchoninic acid (BCA) assay protein kit (Interchim, Montluçon, France), as recommended by the supplier.

2.9. Mass spectrometry

Peptides obtained from purification were analyzed on a LTQ Velos Orbitrap (Thermo Fisher Scientific, Waltham, MA, USA), in infusion mode with an electrospray source in a positive ionization mode. Molecules were infused (5 µl/min) with a 500 µl syringe and analyzed in the Orbitrap (trapped) in full ion scan mode with a mass range of 400-1800 *m/z*. For identification and amino acids sequencing in MS-MS, two types of fragmentation were used: collision-induced dissociation (CID) analyzed by linear trapped quadrupole (LTQ) and Orbitrap, and higher-energy C-trap dissociation (HCD) analyzed by LTQ. Bacteriocins were identified using Proteome Discoverer 1.3 software (Thermo Fisher Scientific) coupled to an in house Mascot search server version 2.4 (Matrix Science, Boston, MA;) and to a Sequest search engine.

2.10. Identification of genes encoding bacteriocin production

The main genes encoding bacteriocin production (Table 2) by *Leuc. mesenteroides* (mesentericins Y105 and B105 and leucocins K, B, A and A-TA33a) were investigated [15]. DNA was extracted using Bacterial DNA commercial kit (Omega Bio-Tek, Norcross, GA, USA), as recommended by the supplier. The DNA concentration was determined at 260-280 nm absorbance values (A_{260}/A_{280}), using spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany). A standard reaction (50 µl final volume) containing 100 ng of DNA, 0.2 µM of each primer (Table 1), 9 µl of 5 x master mix (BioFidal, Vaulx-En-Velin, France) and 36 µl of sterile bi-distilled water was used. The amplification program (35 cycles) was carried out in a thermocycler Touchgene gradient (Techne, Stone, Staffordshire, UK) and was composed by denaturation step at 95 °C for 1 min, annealing at 45 °C for 45 s and extension at 72 °C for 1.5 min. PCR amplification products were analyzed by electrophoresis (100 V) on agarose gel (1 %, w/v) with 0.5 mg/ml of ethidium bromide in TAE buffer (0.5 x, pH 8.0, Tris-Acetate-EDTA) and observed under ultraviolet light. The sequencing of amplified fragments was carried out by

MilleGen sequencing service (Labège, France), and compared with other DNA sequences presented in the Genebank using BLAST.

Table 1 Specific primers and genes encoding bacteriocin production by *Leuconostoc mesenteroides*

Primers[a]	Sequences (5'–3')	Target genes	Accession numbers
mesBF	ATGCAAGATAAAACAAAA		
mesBR	TTATTTGTGGTTCTTG	*mesB*	AF143443
mesYF	ATGACGAATATGAAGTC		
mesYR	TTACCAAAATCCATTTCC	*mesY*	X81803
lcnKF	ATGAAAAAATTCAAAGAAC		
lcnKR	TTAATTGTTAATGGTTGAAG	*lcnK*	AF420260
lcnAF	ATGATGAACATGAAACCTAC		
lcnAR	TTACCAGAAACCATTTCC	*lcnA*	M64371
lcnBF	ATGAATAACATGAAATCTGC		
lcnBR	TTACCAGAAACCATTTCCACC	*lcnB*	S72922
lcnA-TAF	TACTACTTGTACTTTGGATG		
lcnA-TAR	TGGTCTTTGGTAAAGGTGG	*lcnA-TA33a*	AF036713

[a] F: forward primer; R: reverse primer.

2.11. Presence of genes encoding virulence factors, vancomycin resistance and biogenic amines production

The investigation of the presence of genes encoding virulence factors, vancomycin resistance and biogenic amines production by *Leuc. mesenteroides* subsp. *mesenteroides* SJRP55 was carried out using the primers and conditions described in Table 2. The amplified products were separated by electrophoresis on 0.8–2.0 % (w/v) agarose gels in 0.5 x TAE buffer. Gels were stained in TAE buffer containing 0.5 µg/ml ethidium bromide (Sigma-Aldrich).

Table 2 Primers sequences used in the investigation of genes encoding virulence factors, vancomycin resistance and biogenic amines production

Targets	Genes	Primer names and sequence 5'→ 3'	Annealing temperature (°C)	Fragment size (bp)	References
Virulence factors	*gelE*[a]	GEL11: TATGACAATGCTTTTTGGGAT GEL12: AGTGCACCCGAAATAATATA	47	213	Vankerchoven et al. 2004
	hyl	HYL n1:ACAGAAGAGCTGCAGGAAATG HYL n2:GACTGACGTCCAAGTTTCCA	53	276	Vankerchoven et al. 2004
	asa1	ASA11: GCACGCTATTACGAACTATGA ASA12: TAAGAAAGAACATCACCACGA	50	375	Vankerchoven et al. 2004
	esp	ESP14F: AGATTTCATCTTTGATTCTTG ESP12R: AATTGATTCTTTAGCATCTGG	47	510	Vankerchoven et al. 2004
	cylA	CYT I: ACTCGGGGATTGATAGGC CYT IIb: GCTGCTAAAGCTGCGCTT	52	688	Vankerchoven et al. 2004
	efaA	EFA-AF: GCCAATTGGGACAGACCCTC EFA-AR: CGCCTTCTGTTCCTTCTTTGGC	57	688	Martin-Platero et al. 2009
	ace	ACE-F: GAATTGAGCAAAAGTTCAATCG ACE-R: GTCTGTCTTTTCACTTGTTTC	48	1008	Martin-Platero et al. 2009
Antibiotic resistance	*vanA*	VAN-AF: TCTGCAATAGAGATAGCCGC VAN-AR: GGAGTAGCTATCCCAGCATT	52	377	Martin-Platero et al. 2009
	vanB	VAN-BF: GCTCCGCAGCCTGCATGGACA VANB-R: ACGATGCCGCCATCCTCCTGC	60	529	Martin-Platero et al. 2009
Biogenic amines	*hdc1*	JV16HC: AGATGGTATTGTTTCTTATG JV17HC: AGACCATACACCATAACCTT	46	367	De Las Rivas et al. 2005
	hdc2	106: AAYTCNTTYGAYTTYGARAARGARG 107: ATNGGNGANCCDATCATYTTRTGNCC	50	534	De Las Rivas et al. 2005
	tdc	P1-rev: CCRTARTCNGGNATAGCRAARTCNGTRTG P2-for: GAYATNATNGGNATNGGNYTNGAYCARG	55	924	De Las Rivas et al. 2005
	odc	3: GTNTTYAAYGCNGAYAARACNTAYTTYGT 16: TACRCARAATACTCCNGGNGGRTANGG	54	1446	De Las Rivas et al. 2005

[a]*gelE* (gelatinase), *hyl* (hyaluronidase), *asa1* (aggregation substance), *esp* (enterococcal surface protein), *cylA* (cytolysin), *efaA* (endocarditis antigen), *ace* (collagen adhesin), *vanA* and *vanB* (vancomycin resistance), *hdc1* and *hdc2* (histidine decarboxylase), *tdc* (tyrosine decarboxylase) and *odc* (ornithidine decarboxylase).

3. Results and Discussion

3.1. Spectrum of antimicrobial activity

Leuc. mesenteroides subsp. *mesenteroides* SJRP55 showed promising results due to its antimicrobial activity against *Enterococcus* spp., *Ent. faecalis, Ent. faecium, Leuc. mesenteroides* subsp. *mesenteroides, Listeria innocua* and *L. monocytogenes* (Table 3) and absence of inhibition against *Lactobacillus* species, which was previously observed with other bacteriocinogenic strains [27,15]. Since *Leuc. mesenteroides* subsp. *mesenteroides* SJRP55 or its antimicrobial substances can inhibit the growth of these microorganisms, they can be employed in dairy food products as co-culture or as partially or fully purified antimicrobial compound(s) [28].

Leuconostoc species can produce class IIa bacteriocins, which have an anti-*Listeria* activity [16]. *Listeria* spp. and particularly *Listeria monocytogenes* is a serious foodborne pathogen that can cause diseases in human. Once this microorganism can grow in different food matrices and environment conditions, its inhibition is very important for food industry. Other studies reported the anti-*Listeria* activity of bacteriocins produced by *Leuconostoc* spp.: *Leuc. carnosum* LA44A isolated from sausages [29], *Leuc. mesenteroides* subsp. *dextranicum* ST99 isolated from cereal-fermented beverage [13], *Leuc. citreum* GJ7 isolated from fermented vegetables [30], *Leuc. mesenteroides* CM135 and CM160 isolated from fruit and vegetables [14], *Leuc. carnosum* IDE1105 isolated from pork meat [31] and *Leuc. mesenteroides* 453Lab isolated from Polish cheese [17].

Leuc. mesenteroides subsp. *mesenteroides* SJRP55 inhibited *Leuc. mesenteroides* subsp. *mesenteroides* UCV10CET. The inhibition of closely related bacteriocinogenic species is very common, as has been reported already for bacteriocins produced by *Leuc. carnosum* LA44A [29], *Leuc. carnosum* F10 [32] and *Leuc. citreum* GJ7 [30]. Moreover, specific immunity mechanisms can protect the same species against bacteriocins produced by other strains.

Table 3 Spectrum of antimicrobial activity of the cell-free supernatant (CFS) of *Leuconostoc mesenteroides* subsp. *mesenteroides* SJRP55

Indicator microorganisms	Growth medium[a]	CFS SJRP55[b]
Enterococcus sp. V1-V20, V28, V31-V39	MRS	8[c]/30[d]
Ent. faecalis V21-V27, V29, V30, V40-V43, ATCC 19443	MRS	6/14
Ent. faecium ET05, ST05, ST211Ch, ET12, ET88	MRS	1/5
Lactobacillus curvatus ET06, ET30, ET31	MRS	0/3
Lact. delbrueckii ET32, B15, B16	MRS	0/3
Lact. delbrueckii subsp. *bulgaricus* B1, B2, B5	MRS	0/3
Lact. fermentum ET35	MRS	0/1
Lact. plantarum AM4, ST216Ch, ST8Sh, ST202Ch, ST16Pa	MRS	0/5
Lact. sakei ST22Ch, ST153Ch, ST154Ch, 2a, ATCC 15521	MRS	0/5
Lactococcus lactis subsp. *lactis* D2-D5, B17	MRS	0/5
Leuc. mesenteroides subsp. *mesenteroides* UCV10CET	MRS	1/1
Leuc. lactis D6	MRS	0/1
Pediococcus acidilactici ET34	MRS	0/1
Listeria innocua ATCC 33090	BHI	1/1
L. monocytogenes 103, 104, 106, 211, 302, 409, 506, 603, 620, 703, 709, 724, ATCC 7644	BHI	8/13

[a] MRS = de Man, Rogosa, Sharpe agar (Difco), BHI = Brain Heart Infusion agar (Difco) at 30 °C for 24 h,
[b] Indicator microorganisms sensitive to CFS of SJRP55 strain,
[c] Inhibited strains,
[d] Total number of strains tested.
Ent. faecalis series V1-V43 from Prof. Dr. Luis Nero, UFV, Viçosa, MG, Brazil
ET series from Prof. Dra. Elisabetta Tome, Universidade Central de Venezuela, Caracas, Venezuela
ST series from Dr. Svetoslav Todorov, USP, Sao Paulo, SP, Brazil.
Listeria monocytogenes from Prof. Dra. Maria Teresa Destro, USP, Sao Paulo, SP, Brazil

3.2. Effect of enzymes, pH, temperature and chemical agents on antimicrobial compounds

The activity of antimicrobial substances produced by *Leuc. mesenteroides* subsp. *mesenteroides* SJRP55 was inhibited by proteolytic enzymes (pepsin, protease of *Aspergillus saitoi*, trypsin and α-chymotrypsin; Table 4). The eventual effect of hydrogen peroxide or glycosylation on antimicrobial activity was eliminated since the activity of antimicrobial compounds produced by *Leuc. mesenteroides* subsp. *mesenteroides* SJRP55 was not modified after treatment with catalase, α-amylase and lipase. These results demonstrated the proteinaceous nature of the antimicrobial activity and suggested that the activity was due to bacteriocins production and that these bacteriocins do not belong to class IV [16], which contains carbohydrates and lipids in their structure. The same results were obtained when experiments were performed on partially purified bacteriocin (Table 4).

The antimicrobial compounds produced by *Leuc. mesenteroides* subsp. *mesenteroides* SJRP55 were stable after incubation at pH 2.0-10.0 and under treatment with different chemical compounds. However, the antimicrobials were inactivated after treatment at pH 12.0 (Table 4). The loss of antimicrobial activity can be attributed to proteolytic degradation, protein aggregation or instability due to extreme pH [33, 34]. Similar results were reported by Mataragas et al. [27] with *Leuc. mesenteroides* L124 and *Lact. curvatus* L442 isolated from fermented sausages, and Batdorj et al. [25] with *Ent. durans* A5-11 isolated from kumis. The same results were obtained when partially purified bacteriocin was treated at different pH values and with chemical agents.

Table 4 Effect of enzymes, pH, temperatures and chemical agents on the antimicrobial substances produced by *Leuconostoc mesenteroides* susbp. *mesenteroides* SJRP55

Treatments	CFS	Partially purified active peptides
α-amylase, lipase, catalase	+	+
pepsin, protease, trypsin, α-chymotrypsin	–	–
pH 2–10	+	+
pH 12	–	–
25, 30, 37, 45, 60, 100 ^{o}C for 1 h	+	+[b]
25, 30, 37, 45, 60, 100 ^{o}C for 2 h	+	+[b]
121 ^{o}C for 20 min	+/–[a]	–
SDS, Tween 20, Tween 80, Triton X-100, Na-EDTA, NaCl (1%) and 1M urea	+	+
Control (pH, enzymes, temperatures, chemical agents)	–	–

+ = inhibition zone (> 2mm); – = no inhibition zone (Todorov et al., 2010)
[a] Decrease of antimicrobial activity at 121 ^{o}C for 20 min
[b] No inhibition was found at 100 ^{o}C 1 h/2 h.

Leuc. mesenteroides subsp. *mesenteroides* SJRP55 produced antimicrobial substances that were resistant to heating up to 100 ºC for 2 h, however a decrease in its activity was observed after a treatment at 121 ºC for 20 min (Table 4). Partially purified bacteriocin was resistant only for treatments at 25, 37, 45 and 60 ºC for 2 h. Probably, the purification steps related with removal of other proteins make it more sensitive than the crude CFS. Bacteriocins produced by *Leuc. mesenteroides* subsp. *mesenteroides* Y105 [10], *Leuc. mesenteroides* CM135 and CM160 [14], *Enterococcus* strains [35] and *Leuc. mesenteroides* 25Lab [17] were also resistant to high temperature treatments. However, we need to keep in consideration that

smallest bacteriocins normally are more thermostable compared with the ones exhibiting higher molecular masses. Thermostability of bacteriocin is an important characteristic in dairy industry, making this compound interesting to be applied in cheese and fermented milk after pasteurization.

3.3. Kinetics of growth of *Leuc. mesenteroides* subsp. *mesenteroides* SJRP55 and the antimicrobial compounds production

The antimicrobial substances produced by *Leuc. mesenteroides* subsp. *mesenteroides* SJRP55 were detected in the beginning of logarithmic phase of growth for both studied temperatures (25 °C and 30 °C, [Fig. 1]), suggesting that these compounds are primary metabolites [17]. This behavior was confirmed by bacteriocins produced by various LAB, such as *Lact. plantarum* ST28MS and ST26MS [36], *Leuc. mesenteroides* 25Lab [17] and *Pediococcus pentosaceus* ST44AM [37].

Maximum active compounds titer was recorded at the end of logarithmic phase until the stationary phase, with antimicrobial activity of 3.200 AU/ml at 25 ºC and 1.600 AU/ml at 30 ºC, determined against *L. monocytogenes* ATCC 7644. The production of active substances may be subject to changes in growth conditions such as temperature, oxygen, water activity, pH and nutrients, which can modify the population density and affect the expression of genes encoding for their production.

In the case of bacteriocins, the increase of its production may be due to a better utilization of energy at low growth rates [19]. Thus, the fact that our compounds presented better production at 25 ºC corroborates with the hyphotesis of bacteriocins production. The temperature also affected the bacteriocin production by *Leuconostoc* spp. [15, 19, 32]. In addition, the logarithmic and stationary phases are the most important periods for the production of bacteriocin, as previously shown in other studies [14, 17]. When the growth was performed at 25 °C, production of active compounds largely decreased at the end of stationary phase (after 45 h). Similar reduction of activity was observed earlier when *Leuc. mesenteroides* subsp.

mesenteroides SJRP55 grew at 30 °C (near 35 h). This can be due to the instability, aggregation and degradation of the peptides by extracellular proteolytic enzymes [24].

The pH values decreased from 6.28 to 4.25 at 25 ºC and from 6.45 to 4.66 at 30 ºC, and the antimicrobial activity was not affected by this decrease of pH, suggesting the stability of the compounds in such conditions. Moreover, in case of bacteriocins production, low pH value can enhance the disintegration of aggregated bacteriocins [32].

During the cultivation period, the OD_{600} values reached a maximum value of 0.833 at 25 ºC and 0.879 at 30 ºC, showing low cellular growth when compared with others studies. However, the antimicrobial activity was unaffected.

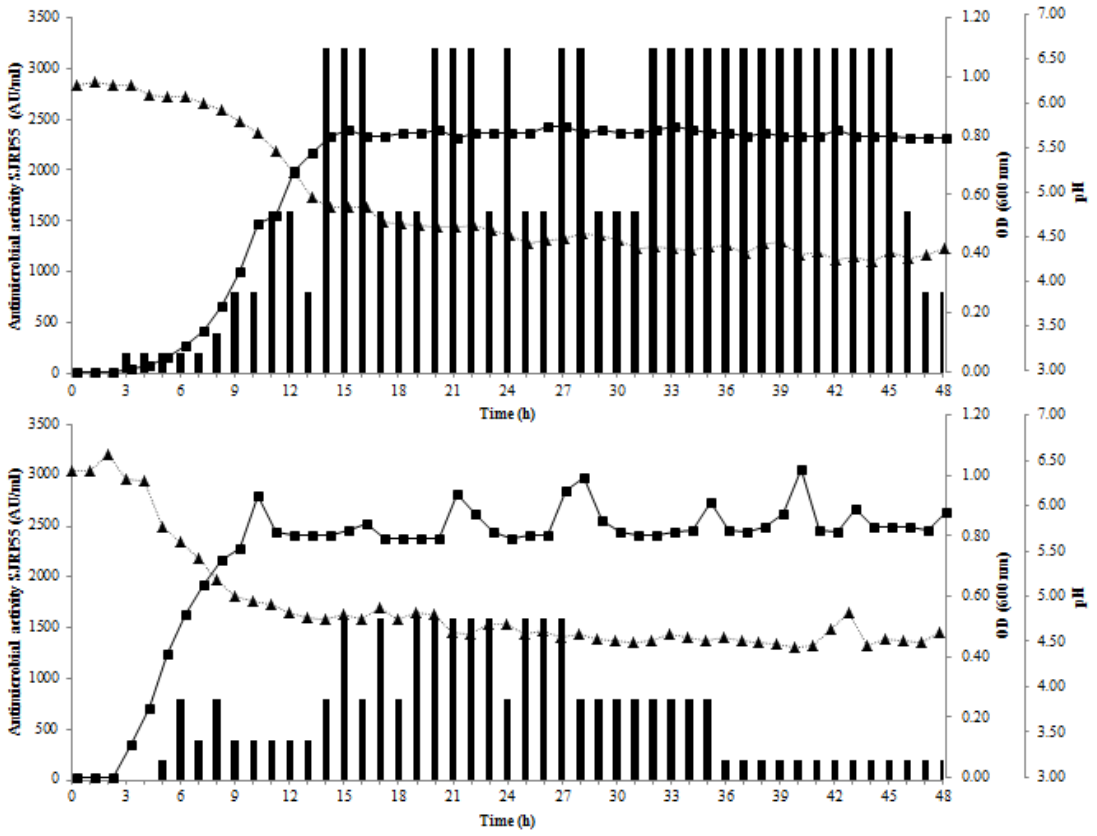


Fig. 1 Kinetics of growth of *Leuconostoc mesenteroides* SJRP55 strain and antimicrobial compounds production in MRS broth at 25 ºC and at 30 ºC. Antimicrobial activity against *Listeria monocytogenes* ATCC 7644 in AU/mL (bars), OD_{600} (■) and pH (▲). Present results are average of the 3 independent repetitions and standard deviation (SD) is not indicated.

3.4. Mode of action

The antimicrobial substances produced by *Leuc. mesenteroides* subsp. *mesenteroides* SJRP55 showed a decrease on the growth of *Leuc. mesenteroides* subsp. *mesenteroides* UCV10CET, *L. innocua* ATCC 33090 and *L. monocytogenes* ATCC 7644 over a period of 9 h (Fig. 2). Bacteriocins produced by *Leuc. mesenteroides* UL5 and *Leuc. mesenteroides* 25Lab presented similar results [6, 17], whereas different strains of *Leuc. mesenteroides* presented bactericidal effect [14]. Leuconocin F10 produced by *Leuc. carnosum* LF10 induced the formation of pores in the cytoplasm membrane, which promoted a rapid release of K^+ from energized *L. innocua* cells [32]. Moreover, mesentericin Y105 neutralized the plasma membrane potential of *L. monocytogenes* and inhibited the transport of leucine and glutamic acid. It also induced an efflux of pre-accumulated amino acids from cells [38]. Consequently, the mode of action is strain-specific.

In the present study, a slight increase in the cell numbers of *L. innocua*, in presence of the antimicrobial compounds produced by SJRP55 was observed at the end of the incubation period. Similar results have been recorded for *Lactobacillus sakei*, *Lactobacillus curvatus* strains after exposure to bacteriocins produced by *Leuc. mesenteroides* L124 and *Lact. curvatus* L442 [27]. However, the responses of different strains within the same species or genera can vary widely [39]. The resistance of *L. monocytogenes* strains to various antimicrobial substances may occur naturally or be acquired [40] and the resistance to class IIa bacteriocins seems to be related to reduced expression of a mannose permease of the phosphotransferase system [28].

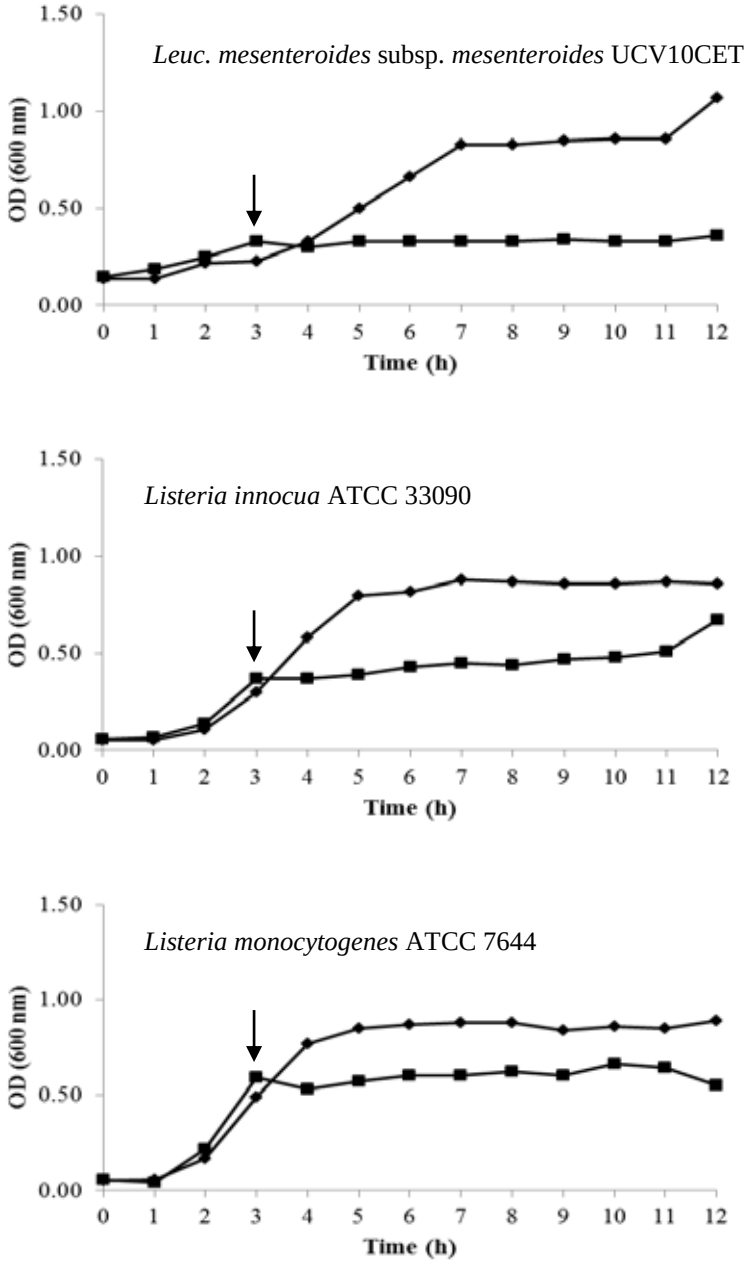


Fig. 2 Mode of action of antimicrobial compounds produced by SJRP55 against *Leuconostoc mesenteroides* subsp. *mesenteroides* UCV10CET, *Listeria innocua* ATCC 33090 and *L. monocytogenes* ATCC 7644. The arrows indicate the time when the cell-free supernatant (CFS) of SJRP55 strain was added (after 3 h of incubation). (■) growth of indicator microorganism in presence of CFS and (♦) growth of indicator microorganism in absence of CFS, shown as optical density (OD) reading values measured at 600nm. Present results are average of the 3 independent repetitions and SD is not indicated.

3.5. Determination of the reduction of viable cell counts of indicator microorganisms in the presence of antimicrobial compounds

The antimicrobial compounds produced by *Leuc. mesenteroides* subsp. *mesenteroides* SJRP55 did not present a strong inhibition in the growth of indicator microorganisms (*Leuc. mesenteroides* subsp. *mesenteroides* UCV10CET, *L. innocua* ATCC 33090 and *L. monocytogenes* ATCC 7644), confirming a reduction on the growth of indicator microorganisms, which was observed in other assays. After contact time of 1 h in presence of SJRP55 bacteriocin, a decrease of 1 log of viable cells of *Leuc. mesenteroides* subsp. *mesenteroides* UCV10CET and a slight reduction of viable cells of *L. innocua* ATCC 33090 and *L. monocytogenes* was measured (Fig. 3). Probably due to the time of contact, the antimicrobials concentration or even its the activity was insufficient to inhibit the indicator microorganisms. In a similar study, the bacteriocinogenic strain *Pediococcus pentosaceus* ST44AM isolated from marula fruit inhibited *L. ivanovii* subsp. *ivanovii* ATCC 19119, *L. innocua* 2030C and *Ent. faecium* HKLHS after 1 h of contact time [37].

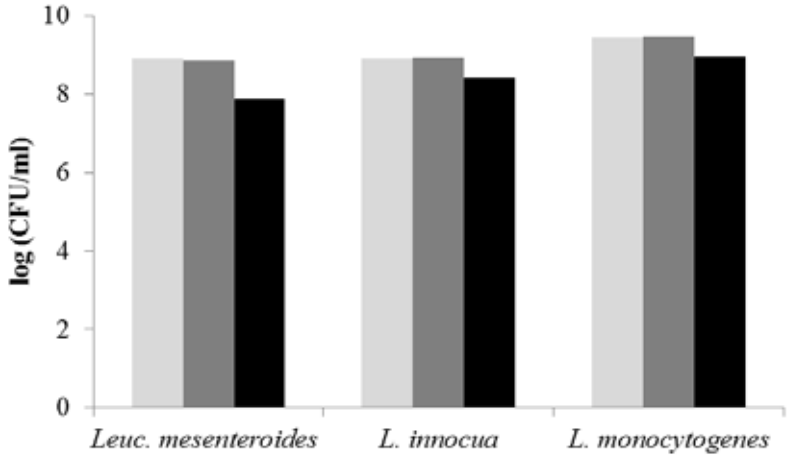


Fig. 3 Reduction of viable cells of *Leuconotoc mesenteroides* subsp. *mesenteroides* UCV10CET, *Listeria innocua* ATCC 33090 and *L. monocytogenes* ATCC 7644 in presence of cell-free supernatant (CFS) of SJRP55 strain; initial population (light gray bar), final population (dark gray bar), and final population in presence of CFS (black bar). Present results are average of the 3 independent repetitions and SD is not indicated.

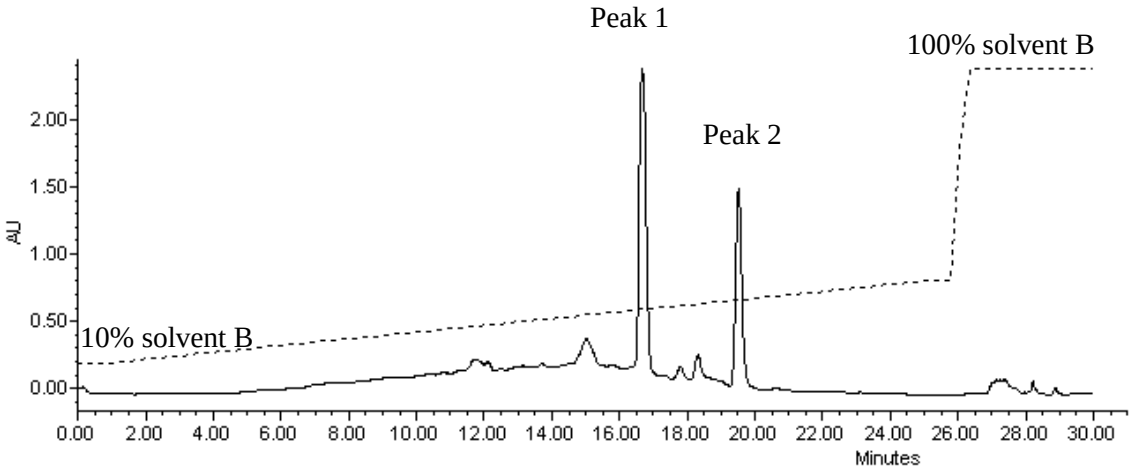
3.6. Adsorption of the active peptides on the producer cells

The antimicrobial peptides produced by SJRP55 strain did not adhere to the producer cells (data not shown). Depending on pH values, the secretion of bacteriocins by producer cells can involve two different processes, adsorption at the surface of cells or excretion into the environment. The molecules are released better at pH 1.5-2.0 than at pH 6.0-7.0, when the bacteriocins are frequently bound to producer cells [41]. These results are in accordance with Mataragas et al. [27], which bacteriocins produced by *Leuc. mesenteroides* L124 and *Lactobacillus curvatus* L442 were adsorbed on the producing bacterial cells. However, treatment of the cells at pH 2.0 did not de adsorb the bacteriocin from the cell surface, compared to the control. Previous studies with *mesenterocin* ST99 [13], bacteriocins produced by *Lact. plantarum* ST194BZ, ST414BZ and ST664BZ, *Lact. pentosus* ST712BZ, *Lact. rhamnosus* ST461BZ and ST462BZ, *Lact. paracasei* ST242BZ and ST284BZ [23] and bacteriocin produced by *Leuc. mesenteroides* 25Lab [17] also showed similar results.

3.7. Purification of the active peptides

Active peptides produced by *Leuc. mesenteroides* subsp. *mesenteroides* SJRP55 were precipitated with 60 % ammonium sulfate, which increased 23-fold the specific activity (Table 5). In affinity column purification step, active compounds were eluted in 6 M urea and NaCl solution (pH 3.0) and the specific activity was increased 283.9-fold, showing that the affinity column retained only a very small amount of supernatant proteins, this behavior have already been reported by mesentericin Y105 [10]. However, purification step using the Sep-Pak C_2 column decreased the specific activity, suggesting that amounts of active compounds were retained on the column. The active peptides applied in RP-HPLC presented two peaks with different activity spectrum (Fig. 4). The first peak (retention time of 16.50 min) inhibited *L. monocytogenes* and the second one (retention time of 19 min) inhibited only *Leuc. mesenteroides* subsp. *mesenteroides*. These results showed that *Leuc. mesenteroides* subsp.

mesenteroides SJRP55 produced at least 2 different active compounds. The production of various bacteriocins with different inhibition targets might be an important tool in food application. In most of purification steps, the specific activity increased and the quantity of non-bacteriocin proteins were reduced. Specific activities (AU/mg) of purified compounds were 23880.60 (peak 1) and 63209.88 (peak 2), which corresponded to an increase of 352.8 and 933.7-fold, respectively (Table 5).



F**ig. 4** Reversed phase chromatography profile of active peptides produced by *Leuconostoc mesenteroides* SJRP55 (peaks 1 and 2) on an analytical RP Nucleosil C_8 (Symmetry 300 Å C_8, 5 µm, 7.8 x 100 mm) equilibrated with solvent A (0.5% solvent B, 95% MilliQ water, 0.03% TFA). Elution was performed at a flow-rate of 2.5 ml/min with a linear gradient from 0 to 60% solvent B (80% acetonitrile, 20% isopropanol and 0.05% TFA) for 30 min.

Table 5 Purification steps of *Leuconostoc mesenteroides* subsp. *mesenteroides* SJRP55 active peptides

Purification steps	Volume (ml)	Protein (µg/ml)	Total protein (mg)	Activity (AU/ml)	Total activity (AU)	Specific activity (AU/mg)	Increase of specific activity (AU/mg)
Supernatant	1000	9.4542	9454.20	640	640000	67.69	1.0
Precipitation in $(NH_4)_2SO_4$	100	3.2822	328.22	5120	512000	1559.93	23.0
Affinity column	322.5	0.0333	10.74	640	206400	19219.22	283.9
Sep-Pak C_2	4.375	0.3357	1.47	2560	11200	7625.86	112.7
C_2 RP-HPLC (peak 1)	1.53125	0.1072	0.16	2560	3920	23880.60	352.8
C_2 RP-HPLC (peak 2)	1.09375	0.0405	0.04	2560	2800	63209.88	933.7

3.8. Mass spectrometry

The molecular mass of peptides purified in the previous step and present in peaks 1 (named mesentericin W-SJRP55) and 2 (named mesentericin Z-SJRP55) were 3868 kDa and 3444 kDa, respectively (Fig. 5). The amino acids sequences showed 100 % homology with mesentericin Y105 (peak 1) and mesentericin B105 (peak 2) produced by *Leuc. mesenteroides* subsp. *mesenteroides* Y105 isolated from goat milk [9,10]. The production of similar bacteriocins by different LAB species is common. Limonet et al. [42] showed the synergic effect of mesenterocins 52A and 52B on the strains susceptible to only one of them. This result might be interesting in food preservation, reducing bacteriocin concentrations and amplifying the activity spectrum [42]. Moreover, the produced mesentericins W-SJRP55 and Z-SJRP55 can be suitable for application as biopreservatives, together with a system of multiple preservative principles (hurdles), in order to extend the shelf-life of water buffalo mozzarella cheese or other dairy products.

The bacteriocins showed high similarity to other bacteriocins produced by *Leuconostoc* species, previously reported. Different environmental matrices, environment conditions and geographical regions did not seem to affect the genes encoding bacteriocins, probably because these genes were very well conserved during the evolution of *Leuconostoc* genus. Most probably some bacteriocin genes are part of complex genetic structure and may have some other functions. Once the bacteriocinogenic bacteria are able to inhibit competing species, the transmission of these genetic elements to daughter cells may help to explain their unique ecology and evolution to preserve these genes [43].

Production of bacteriocins by *Leuc. mesenteroides* has been reported by other studies: mesenterocin 5 [6]; leucocin A-UAL 187 [8]; mesentericin Y105 and mesentericin B105 [9,10]; mesenterocin 52A and 52B [12]; leucocin A- B- C-TA33a [11]; mesenterocin ST99 [13]; leucocin C [7]; mesenterocin E131 [15]. However, according to our knowledge, this is the first time that a bacteriocinogenic *Leuc. mesenteroides* subsp. *mesenteroides* has been isolated from water buffalo mozzarella cheese.

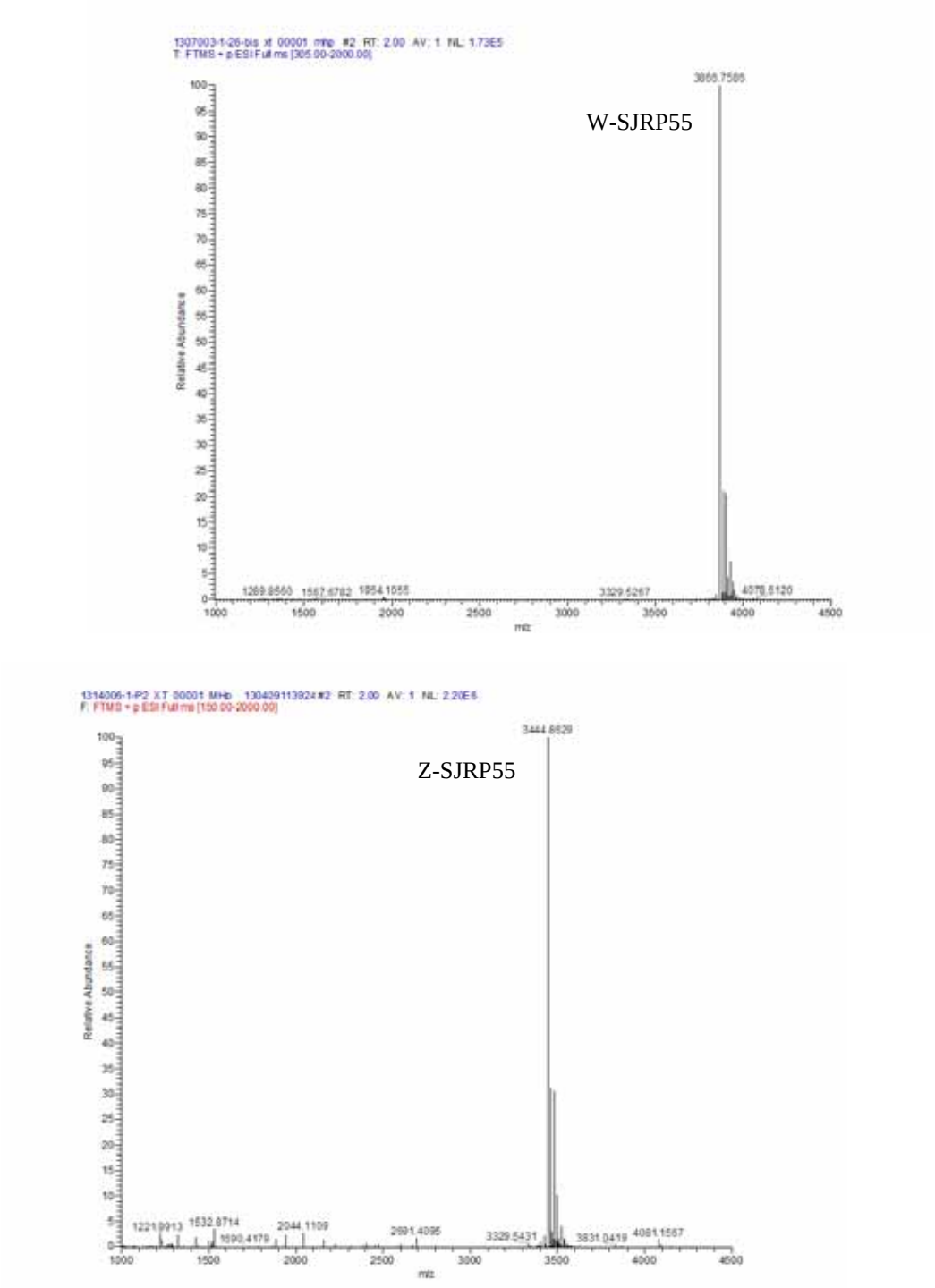


Fig. 5 Mass spectrometry (ESI-MS-MS) of mesentericin W-SJRP55 and mesentericin Z-SJRP55.

3.9. Identification of genes encoding bacteriocin production

DNA from *Leuc. mesenteroides* subsp. *mesenteroides* has been evaluated by PCR with specific primers targeting mesentericins Y105 and B105, leucocins K, A, B and A-TA33a (Table 2). PCR reactions generated only positive results for mesentericin Y105 and mesentericin B105 genes, and after sequencing, a 100 % homology has been found in comparison to already deposited sequences in Genebank [14]. Previous studies have showed that *Leuconostoc* strains also harbored the genes encoding mesentericin Y105 and mesentericin B105 [44], whereas *Leuc. mesenteroides* E131 was able to produce only mesentericin Y105, which was named by the authors mesenterocin E131.

3.10. Presence of genes encoding virulence factors, vancomycin resistance and biogenic amines production

Based on the results of PCR targeting the presence of various virulence factors, biogenic amines and vancomycin resistance genes, *Leuc. mesenteroides* subsp. *mesenteroides* SJRP55 generated positive results for presence of enterococcal surface protein (*esp*), adhesion of collagen (*ace*) and vancomycin resistance (*vanA*) (data not shown).

Enterococcal surface protein gene (*esp*) is a virulence factor specific for *Enterococcus* spp. encoded by the chromosomal *esp* gene, producing a cell wall-associated protein with a structure that includes a central core consisting of distinct tandem repeated units, which is involved in immune evasion [45, 46]. It was proposed that Esp protein may play a role similar to that of the fimbriae of *Escherichia coli* in serving as a colonization factor to adhere and for biofilm formation [47,48]. Moreover, the *esp* gene is found at high frequency in infection-derived isolates, such as endocarditis and urinary tract infection [47].

Transferable resistance genes, especially those located on transposons or plasmid DNA, may pose a risk, since they can be transferred to pathogenic bacteria [49, 50]. Previous studies have reported that *Leuconostoc* spp. has an intrinsic resistance to vancomycin [45] due to

particular characteristics of its cell wall containing D-lactate instead of a D-alanine in the peptidoglycan [51]. According to previous studies, *Leuconostoc* spp. isolated from different sources also showed vancomycin-resistance [52, 53]. Natural (horizontal) gene transfer can happen [54], but it has been seldom confirmed [51]. In addition, no case of infection by consumption of dairy products containing *Leuconostoc* spp. has been described, which allows this microorganism to be considered as safe.

Collagen adhesin gene (*ace*) is another virulence gene commonly found in *Enterococcus* spp. This gene encodes an adhesion protein to collagen that contributes to infective endocarditis and urinary tract infection [55,56].

The *esp* and *ace* genes present in *Leuc. mesenteroides* subsp. *mesenteroides* SJPRP55 can be linked with horizontal transference by *Enterococcus* genus, which is commonly present in many cheeses. On the other hand, the adhesion properties of *esp* and *ace* genes can be an important characteristic in food application or as potential probiotic culture. According to the literature, the presence of these genes is uncommon in *Leuconostoc* genus. Further studies should be done to verify if these virulence genes are being expressed.

We need to keep in consideration that presence of biogenic amines in food products is undesirable, since this can have toxicological effects on humans and animals [57]. However, another sensitive point is the fact that sometimes, production of these biogenic amines can be related with the genes that may be expressed only under specific bacterial growth conditions. Even if the genes are present on the genome, and not expressed in the studied conditions, we need to consider this bacterium as a potential reservoir of the biogenic amines production, or spread of this genes to the other bacteria via horizontal gene transfer. No evidences for the presence of histidine decarboxylase, tyrosine decarboxylase and ornithine decarboxylase encoding genes (*hdc1*, *hdc2*, *tdc*, *odc*, respectively) were found on DNA from *Leuc. mesenteroides* subsp. *mesenteroides* SJPRP55. The absence of virulence and biogenic amines genes is very important for industrial applications of new strains.

4. Conclusion

The activity against *L. monocytogenes* and the resistance under different conditions are interesting characteristics shown by bacteriocins produced by *Leuc. mesenteroides* subsp. *mesenteroides* SJRP55. The purified mesentericin W-SJRP55 and mesentericin Z-SJRP55 can be useful for application as biopreservatives combined with a system of multiple preservative principles (hurdles), in order to extend the shelf-life of water buffalo mozzarella cheese or other dairy products.

Acknowledgments

The authors are grateful to the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Brazil, Project N° 2010/09302-1), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil) for the financial support, to Dra. Elisabetta Tome from Universidad Central de Venezuela, Caracas, Venezuela, for providing *Leuc. mesenteroides* subsp. *mesenteroides* UCV10CET strain (CDCH Project N° PG-03.7371.2008). *Enterococcus* spp. strains used in this study as test organisms in determination of the antibacterial spectrum of activity have been provided by Prof. Dr. Luis Nero from UFV, Viçosa, MG, Brazil.

Conflict of interest

The authors declare that there is no conflict of interest.

References

1. De Vuyst L, Leroy F (2007) Bacteriocins from lactic acid bacteria: production, purification, and food applications. J Mol Microbiol Biotech 13 (4):194-199. doi:10.1159/000104752
2. Adimpong DB, Nielsen DS, Sorensen KI, Derkx PM, Jespersen L (2012) Genotypic characterization and safety assessment of lactic acid bacteria from indigenous African fermented food products. BMC Microbiol 12:75. doi:10.1186/1471-2180-12-75
3. Dobson A, Cotter PD, Ross RP, Hill C (2012) Bacteriocin production: a probiotic trait? Appl Environ Microbiol 78 (1):1-6. doi:10.1128/AEM.05576-11

4. Masuda Y, Ono H, Kitagawa H, Ito H, Mu F, Sawa N, Zendo T, Sonomoto K (2011) Identification and characterization of leucocyclicin Q, a novel cyclic bacteriocin produced by *Leuconostoc mesenteroides* TK41401. Appl Environ Microbiol 77 (22):8164-8170. doi:10.1128/AEM.06348-11

5. Zouhir A, Hammami R, Fliss I, Hamida JB (2010) A new structure-based classification of gram-positive bacteriocins. Protein J 29 (6):432-439. doi:10.1007/s10930-010-9270-4

6. Daba H, Pandian S, Gosselin JF, Simard RE, Huang J, Lacroix C (1991) Detection and activity of a bacteriocin produced by *Leuconostoc mesenteroides*. Appl Environ Microbiol 57 (12):3450-3455

7. Fimland G, Sletten K, Nissen-Meyer J (2002) The complete amino acid sequence of the pediocin-like antimicrobial peptide leucocin C. Biochem Biophys Res Commun 295 (4):826-827

8. Hastings JW, Stiles ME, von Holy A (1994) Bacteriocins of leuconostocs isolated from meat. Int J Food Microbiol 24 (1-2):75-81

9. Héchard Y, Berjeaud JM, Cenatiempo Y (1999) Characterization of the mesB gene and expression of bacteriocins by *Leuconostoc mesenteroides* Y105. Curr Microbiol 39 (5):265-269

10. Héchard Y, Derijard B, Letellier F, Cenatiempo Y (1992) Characterization and purification of mesentericin Y105, an anti-*Listeria* bacteriocin from *Leuconostoc mesenteroides*. J Gen Microbiol 138 (12):2725-2731

11. Papathanasopoulos MA, Revol-Junelles A-M, Lefebvre G, Le Caer JP, Von Holy A, Hastings JW (1997) Multiple bacteriocin production by *Leuconostoc mesenteroides* TA33a, and other *Leuconostoc*/*Weisella* strains. Curr Microbiol 6 (35):331–335

12. Revol-Junelles AM, Mathis R, Krier F, Fleury Y, Delfour A, Lefebvre G (1996) *Leuconostoc mesenteroides* subsp. *mesenteroides* FR52 synthesizes two distinct bacteriocins. Lett Appl Microbiol 23 (2):120-124

13. Todorov SD, Dicks LM (2004) Characterization of mesentericin ST99, a bacteriocin produced by *Leuconostoc mesenteroides* subsp. *dextranicum* ST99 isolated from boza. J Ind Microbiol Biotechnol 31 (7):323-329. doi:10.1007/s10295-004-0153-6

14. Trias R, Badosa E, Montesinos E, Baneras L (2008) Bioprotective *Leuconostoc* strains against *Listeria monocytogenes* in fresh fruits and vegetables. Int J Food Microbiol 127 (1-2):91-98. doi:10.1016/j.ijfoodmicro.2008.06.011

15. Xiraphi N, Georgalaki M, Rantsiou K, Cocolin L, Tsakalidou E, Drosinos EH (2008) Purification and characterization of a bacteriocin produced by *Leuconostoc mesenteroides* E131. Meat Sci 80 (2):194-103. doi:10.1016/j.meatsci.2007.11.020

16. Klaenhammer TR (1993) Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol Rev 12 (1-3):39-85

17. Sip A, Więckowicz M, Olejnik-Schmidt A, Grajek W (2012) Anti-*Listeria* activity of lactic acid bacteria isolated from golka, a regional cheese produced in Poland. Food Control 26 (1):117-124. doi:10.1016/j.foodcont.2012.01.014

18. Nieto-Arribas P, Sesena S, Poveda JM, Palop L, Cabezas L (2010) Genotypic and technological characterization of *Leuconostoc* isolates to be used as adjunct starters in

Manchego cheese manufacture. Food Microbiol 27 (1):85-93. doi:10.1016/j.fm.2009.08.006

19. Drosinos EH, Mataragas M, Metaxopoulos J (2006) Modeling of growth and bacteriocin production by *Leuconostoc mesenteroides* E131. Meat Sci 74 (4):690-696. doi:10.1016/j.meatsci.2006.05.022

20. Gálvez A, Abriouel H, Benomar N, Lucas R (2010) Microbial antagonists to food-borne pathogens and biocontrol. Curr Opin Biotechnol 21 (2):142-148. doi:10.1016/j.copbio.2010.01.005

21. Silva LF (2010) Identificação e caracterização da microbiota lática isolada de queijo mussarela de búfala. Universidade Estadual Paulista, São José do Rio Preto

22. Schillinger U, Lücke FK (1989) Antibacterial activity of *Lactobacillus sakei* isolated from meat. Appl Environ Microbiol 55 (8):1901-1906

23. Todorov SD, Dicks LM (2006) Parameters affecting the adsorption of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum* 423 isolated from sorghum beer. Biotechnol J 1 (4):405-409. doi:10.1002/biot.200500026

24. Todorov SD, Wachsman M, Tome E, Dousset X, Destro MT, Dicks LM, Franco BD, Vaz-Velho M, Drider D (2010) Characterisation of an antiviral pediocin-like bacteriocin produced by *Enterococcus faecium*. Food Microbiol 27 (7):869-879. doi:10.1016/j.fm.2010.05.001

25. Batdorj B, Dalgalarrondo M, Choiset Y, Pedroche J, Metro F, Prevost H, Chobert JM, Haertle T (2006) Purification and characterization of two bacteriocins produced by lactic acid bacteria isolated from Mongolian airag. J Appl Microbiol 101 (4):837-848. doi:10.1111/j.1365-2672.2006.02966.x

26. Yang R, Johnson MC, Ray B (1992) Novel method to extract large amounts of bacteriocins from lactic acid bacteria. Appl Environ Microbiol 58 (10):3355-3359

27. Mataragas M, Metaxopoulos J, Drosinos EH (2002) Characterization of two bacteriocins produced by *Leuconostoc mesenteroides* L124 and *Lactobacillus curvatus* L442, isolated from dry fermented sausages. World J Microbiol Biotechnol 18 (9):847–856

28. Cotter PD, Hill C, Ross RP (2005) Bacteriocins: developing innate immunity for food. Nat Rev Microbiol 3 (10):777-788. doi:10.1038/nrmicro1273

29. van Laack RL, Schillinger U, Holzapfel WH (1992) Characterization and partial purification of a bacteriocin produced by *Leuconostoc carnosum* LA44A. Int J Food Microbiol 16 (3):183-195

30. Chang JY, Lee HJ, Chang HC (2007) Identification of the agent from *Lactobacillus plantarum* KFRI464 that enhances bacteriocin production by *Leuconostoc citreum* GJ7. J Appl Microbiol 103 (6):2504-2515. doi:10.1111/j.1365-2672.2007.03543.x

31. Hartmann HA, Wilke T, Erdmann R (2011) Efficacy of bacteriocin-containing cell-free culture supernatants from lactic acid bacteria to control *Listeria monocytogenes* in food. Int J Food Microbiol 146 (2):192-199. doi:10.1016/j.ijfoodmicro.2011.02.031

32. Parente E, Moles M, Ricciardi A (1996) Leucocin F 10, a bacteriocin from *Leuconostoc carnosum*. Int J Food Microbiol 33 (2-3):231-243

33. Aasen IM, Moretro T, Katla T, Axelsson L, Storro I (2000) Influence of complex nutrients, temperature and pH on bacteriocin production by *Lactobacillus sakei* CCUG 42687. Appl Microbiol Biotechnol 53 (2):159-166

34. de Vuyst L, Callewaert R, Crabbé K (1996) Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin production under unfavourable growth conditions. Microbiology 142 (2):817-827

35. Simova ED, Beshkova DB, Dimitrov Zh P (2009) Characterization and antimicrobial spectrum of bacteriocins produced by lactic acid bacteria isolated from traditional Bulgarian dairy products. J Appl Microbiol 106 (2):692-601. doi:10.1111/j.1365-2672.2008.04052.x

36. Todorov SD, Dicks LM (2005) Characterization of bacteriocins produced by lactic acid bacteria isolated from spoiled black olives. J Bas Microbiol 45 (4):312-322. doi:10.1002/jobm.200410532

37. Todorov SD, Dicks LM (2009) Bacteriocin production by *Pediococcus pentosaceus* isolated from marula (*Scerocarya birrea*). Int J Food Microbiol 132 (2-3):117-126. doi:10.1016/j.ijfoodmicro.2009.04.010

38. Maftah A, Renault D, Vignoles C, Hechard Y, Bressollier P, Ratinaud MH, Cenatiempo Y, Julien R (1993) Membrane permeabilization of *Listeria monocytogenes* and mitochondria by the bacteriocin mesentericin Y105. J Bacteriol 175 (10):3232-3235

39. Papagianni M, Avramidis N, Filioussis G, Dasiou D, Ambrosiadis L (2006) Determination of bacteriocin activity with bioassays carried out on solid and liquid substrates: assessing the factor "indicator microorganism". Microbial Cell Factories 30 (5):1-14

40. Ennahar S, Deschamps N, Richard J (2000) Natural variation in susceptibility of *Listeria* strains to class IIa bacteriocins. Curr Microbiol 41 (1):1-4

41. Ray B (2004) Fundamental Food microbiology CRC Press, New York

42. Limonet M, Revol-Junelles AM, Cailliez-Grimal C, Milliere JB (2004) Synergistic mode of action of mesenterocins 52A and 52B produced by *Leuconostoc mesenteroides* subsp. *mesenteroides* FR 52. Curr Microbiol 48 (3):204-207. doi:10.1007/s00284-003-4165-7

43. Inglis RF, Bayramoglu B, Gillor O, Ackermann M (2013) The role of bacteriocins as selfish genetic elements. Biology Lett 18 (9):1-4

44. Abriouel H, Martin-Platero A, Maqueda M, Valdivia E, Martinez-Bueno M (2008) Biodiversity of the microbial community in a Spanish farmhouse cheese as revealed by culture-dependent and culture-independent methods. Int J Food Microbiol 127 (3):200-208. doi:10.1016/j.ijfoodmicro.2008.07.004

45. Muñoz-Atienza E, Gómez-Sala B, Araújo C, Campanero C, Del Campo R, Hernández PE, Carmen H, Luis MC (2013) Antimicrobial activity, antibiotic susceptibility and virulence factors of lactic acid bacteria of aquatic origin intended for use as probiotics in aquaculture. BMC Microbiology 13 (1):1-22.

46. Vankerckhoven V, Van Autgaerden T, Vael C, Lammens C, Chapelle S, Rossi R, Jabes D, Goossens H (2004) Development of a multiplex PCR for the detection of asa1, gelE, cylA, esp, and hyl genes in enterococci and survey for virulence determinants among European hospital isolates of Enterococcus faecium. J Clin Microbiol 42 (10):4473-4479. doi:10.1128/JCM.42.10.4473-4479.2004

47. Fisher K, Phillips C (2009) The ecology, epidemiology and virulence of *Enterococcus*. Microbiology 155 (Pt 6):1749-1757. doi:10.1099/mic.0.026385-0

48. Shankar N, Lockatell CV, Baghdayan AS, Drachenberg C, Gilmore MS, Johnson DE (2001) Role of *Enterococcus faecalis* surface protein Esp in the pathogenesis of ascending urinary tract infection. Infect and Immun 69 (7):4366-4372. doi:10.1128/IAI.69.7.4366-4372.2001

49. Aymerich T, Martin B, Garriga M, Vidal-Carou MC, Bover-Cid S, Hugas M (2006) Safety properties and molecular strain typing of lactic acid bacteria from slightly fermented sausages. J Appl Microbiol 100 (1):40-49. doi:10.1111/j.1365-2672.2005.02772.x

50. Devirgiliis C, Barile S, Perozzi G (2011) Antibiotic resistance determinants in the interplay between food and gut microbiota. Genes Nutr 6 (3):275-284. doi:10.1007/s12263-011-0226-x

51. Hemme D, Foucaud-Scheunemann C (2004) *Leuconostoc*, characteristics, use in dairy technology and prospects in functional foods. Int Dairy J 14 (6):467-494. doi:10.1016/j.idairyj.2003.10.005

52. Aswathy RG, Ismail B, John RP, Nampoothiri KM (2008) Evaluation of the probiotic characteristics of newly isolated lactic acid bacteria. Appl Biochem Biotechnol 151 (2-3):244-255. doi:10.1007/s12010-008-8183-6

53. Shobharani P, Agrawal R (2011) A potent probiotic strain from cheddar cheese. Indian J Microbiol 51 (3):251-258. doi:10.1007/s12088-011-0072-y

54. Dicks LMT, Todorov SD, Franco BDGM (2009) Current status of antibiotic resistance in lactic acid bacteria In: Bonilla AR, Muniz KP (eds) Antibiotic resistance: causes and risk factors. Nova Publisher, New York, pp 1-46

55. Łysakowska ME, Denys A, Sienkiewicz M (2012) Frequency of ace, epa and elrA Genes in clinical and environmental strains of *Enterococcus faecalis*. Indian J Microbiol 52 (4):612-616. doi:10.1007/s12088-012-0285-8

56. Nallapareddy SR, Singh KV, Sillanpaa J, Zhao M, Murray BE (2011) Relative contributions of Ebp Pili and the collagen adhesin ace to host extracellular matrix protein adherence and experimental urinary tract infection by *Enterococcus faecalis* OG1RF. Infect Immun 79 (7):2901-2910. doi:10.1128/IAI.00038-11

57. de Las Rivas B, Marcobal A, Munoz R (2005) Improved multiplex-PCR method for the simultaneous detection of food bacteria producing biogenic amines. FEMS Microbiol Lett 244 (2):367-372. doi:10.1016/j.femsle.2005.02.012

Capítulo IV

Conclusão geral

CONCLUSÃO GERAL

A cepa *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 apresentou propriedade probiótica promissora e produção de bacteriocinas, com importante destaque para as características:

- Presença de perfil probiótico desejável em testes *in vitro*, viabilizando a continuidade de estudos *in vivo*;
- Viabilidade durante o período de estocagem dos leites fermentados, além de não apresentar atividade inibitória sobre *Streptococcus thermophilus* TA040*;*
- Produção de bacteriocinas, identificadas como mesentericina Y105 e mesentericina B105, com atividade bacteriostática sobre *L. monocytogenes* e resistência a diferentes condições de estresse.

Os resultados obtidos mostraram o potencial probiótico *in vitro* da cultura *Lc. mesenteroides* subsp. *mesenteroides* SJRP55 e a aplicação promissora como cultura produtora de bioconservante em leites fermentados. As bacteriocinas purificadas também podem ser aplicadas como uma barreira tecnológica para a conservação de alimentos.

ANEXO I

Estudos complementares realizados durante o doutorado

O projeto inicial de doutorado intitulado "Caracterização da atividade antifúngica de bactéria lática comercial e selvagem sobre levedura isolada de bebida láctea fermentada", recebeu financiamento da Fundação de Amparo à Pesquisa e Desenvolvimento (FAPESP) durante o período de dois anos. No primeiro ano de execução do projeto, os resultados encontrados não foram satisfatórios, pois a cepa comercial probiótica *Lactobacillus acidophilus* (La-5), produtora de substância antifúngica, apresentou variações na produção do composto inibidor impossibilitando a continuidade do trabalho. Dadas as dificuldades encontradas, no segundo ano de doutorado foram selecionadas aleatoriamente 38 cepas de bactérias láticas isoladas de mussarela de búfala pertencentes ao banco de culturas do Laboratório de Leites e Derivados-UNESP/IBILCE. Foi feito um *screening* para avaliar a produção de substâncias antimicrobianas, o qual foi realizado no Laboratório de Microbiologia da FCF-USP/SP. Das 38 cepas analisadas, somente seis cepas apresentaram atividade antimicrobiana frente a diferentes cepas de *Listeria monocytogenes* e *L. innocua,* e sobre espécies filogeneticamente relacionadas. Durante o estudo realizado no Laboratório de Leites e Derivados – UNESP/IBILCE foi avaliada a natureza do composto inibidor (Capítulo III) produzida por essas seis cepas, porém somente três apresentaram estabilidade na produção do composto. Assim, foi dada a continuidade nos estudos utilizando-se somente estas cepas, as quais foram identificadas pelo sequenciamento do gene 16S rRNA como *Leuconostoc mesenteroides* subsp. *mesenteroides*. Foi realizada a técnica de RAPD-PCR utilizando três primers diferentes para verificar a homologia no DNA ribossomal. Os resultados mostraram que as cepas eram geneticamente diferentes, porém com produção semelhante do composto inibidor. Assim, foi selecionada somente uma cepa para dar continuidade à purificação da bacteriocina produzido por esse micro-organismo. A purificação foi realizada na L'Ecole Nationale Vétérinaire, Agroalimentaire et de l'Alimentation e no Institut National de la Recherche Agronomique, em Nantes, França por um período de seis meses. A avaliação do perfil probiótico da cepa e sua viabilidade em leites fermentados ao longo do período de estocagem foram ambos realizados no Laboratório de Leites e Derivados – UNESP/IBILCE.