UNIVERSIDADE DE SÃO PAULO

FACULDADE DE CIÊNCIAS FARMACÊUTICAS DE RIBEIRÃO PRETO

UNIVERSITÉ DE BRETAGNE OCCIDENTALE

ÉCOLE DOCTORALE SANTÉ, INFORMATION-COMMUNICATION, MATHEMATIQUES, MATIÈRES

Isolation of lactic acid bacteria from milk and cheese with potential for food biopreservation and utilization for increasing whey digestibility

Fabrício Luiz Tulini

Ribeirão Preto

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Candidate: Fabrício Luiz Tulini

Brazilian Supervisor: Prof. Dr. Elaine Cristina Pereira De Martinis

French supervisor: Prof. Dr. Gwenaelle Le Blay

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Preface

This thesis was done in a co-tutelle agreement between the Faculdade de Ciências Farmacêuticas de Ribeirão Preto - Universidade de São Paulo (FCFRP - USP, Ribeirão Preto, Brazil) and the École Doctorale Santé, Information-Communication, Mathematiques, Matières - Université de Bretagne Occidentale (EDSICMA - UBO, Brest, France). This project is inserted in the collaborative program CAPES-COFECUB 730/11 (Coordination for the Improvement of Higher Education Personnel - Brazil, and the French Committee for the Evaluation of Academic and Scientific Cooperation with Brazil) entitled “Proteínas bioativas de bactérias láticas no controle da alergenicidade e digestibilidade do lactosoro e da multiplicação de microrganismos patogênicos em produtos lácteos” / “Bactéries lactiques - Le contrôle d’allergénicité, de la digestibilité du lactosérum et l’inhibition des pathogènes”, coordinated by Prof. Dr. Bernadette D. G. M. Franco (FCF-USP, Brazil) and Prof. Dr. Thomas Haertlé (INRA, France). The experiments were performed at the USP, UBO and INRA Angers-Nantes.

The text is divided in seven parts. In the first chapter (“Introduction”), a brief bibliographical review is presented based on mainly topics of the thesis. In the second chapter, the objectives are presented.

In chapter 3, the isolation, identification and preliminary characterization of lactic acid bacteria (LAB) from Brazilian dairy samples with antimicrobial and proteolytic activities are presented. Based on antimicrobial and proteolytic activities, the most active isolates were selected for further studies. The results of this part of the project derived the manuscript “Screening for antimicrobial and proteolytic activities of lactic acid bacteria isolated from cow, buffalo and goat milk and cheese in southeast region of Brazil”, submitted for publication.

In chapter 4, a study on proteolytic activity of selected LAB is reported. Proteolytic activity of LAB on milk proteins was evaluated under different conditions, and hydrolyzed milk proteins were analyzed by high-performance liquid chromatography. These analyses were performed at the INRA Angers-Nantes (Institut National de la Recherche Agronomique) in Nantes, France, under supervision of Prof. Dr. Thomas Haertlé. A manuscript was written with these results and is going to be submitted to a scientific journal.

In chapter 5, it was evaluated the immunomodulatory effect of the fermented milk obtained from LAB (as shown in chapter 4), as a source of hydrolyzed milk proteins. The supernatant of fermented milk was tested in cultures of monocytes, and it was evaluated the differentiation into macrophages and TNF-α production. This study was done at the UBO in Brest, France. A manuscript based on this chapter will also be submitted for publication to a scientific journal.

In chapter 6, it was evaluated the probiotic potential of an antimicrobial LAB described in chapter 3 (*Lactobacillus paraplantarum* FT259) and a proteolytic LAB described in chapters 4 and 5 (*Lactobacillus paracasei* FT700). They were evaluated with regard to survival at low pH, in the presence of digestive enzymes and bile salts. The antibiotic resistance profile was determined and the antimicrobial peptide produced by one of the strains was partially purified and characterized. This study was realized at the FCFRP-USP in Ribeirão Preto, Brazil, and part of the results were published as a manuscript entitled “Identification and evaluation of the probiotic potential of *Lactobacillus paraplantarum* FT259, a bacteriocinogenic strain isolated from Brazilian semi-hard artisanal cheese” (TULINI, F. L.; WINKELSTRÖTER, L. K.; DE MARTINIS, E. C. P. Anaerobe, v. 22, p. 57-63, 2013).

Finally, the chapter 7 presents the closing remarks concerning all results obtained during the thesis.

ABSTRACT

TULINI, F. L. **Isolation of lactic acid bacteria from milk and cheese with potential for food biopreservation and utilization for increasing whey digestibility**. 2014. 182 p. Doctoral Thesis. Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto (Brazil), and École Doctorale Santé, Information-Communication, Mathematiques, Matières – Université de Brest, Brest (France), 2014.

Lactic acid bacteria (LAB) have been used by mankind for centuries due to their technological properties and ability to improve sensorial aspects of foods. Moreover, it is increasingly the demand of consumers for food with high quality and safety. LAB have many properties for food applications. One of the main properties of these bacteria is the production of substances that inhibit the growth of food-borne pathogens and spoilage microorganisms, such as organic acids, H_2O_2 and bacteriocins. The latter are defined as antimicrobial peptides ribosomally produced by some bacteria with a narrow inhibitory spectrum. Bacteriocins may reduce the growth of undesirable bacteria and increase food safety and improve shelf-life. These inhibitory substances produced by LAB may also inhibit fungi that are largely responsible for food deterioration. Another important property of LAB is the ability to modify milk proteins during fermentation processes, which can contribute to modify the allergenic potential of milk proteins and lead to production of bioactive peptides. It has been shown that peptides derived from milk protein hydrolysis may have different biological activities, such as antihypertensive, antioxidant, antimicrobial and immunomodulatory. In summary, LAB have many application in food safety and technology, encouraging the search for new strains. In the present study, LAB with antimicrobial and/or proteolytic activities were isolated from cow, buffalo and goat milk and cheese obtained in the southeast region of Brazil. From 156 milk and cheese samples, 815 isolates were obtained on selective agars for lactic acid bacteria (LAB). The majority of isolates was Gram-positive (cocci or bacilli) and negative for catalase production. These new isolates were evaluated for antimicrobial activity using agar antagonism tests (spot-on-the-lawn assay), and also for proteolytic activity on milk proteins. The most proteolytic isolates were chosen for cultivation in skim milk followed by analysis using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to confirm the proteolysis in fermented milk. Four out of 815 isolates tested produced bacteriocins and were identified as *Lactobacillus paraplantarum* FT 259 (one strain) and *Streptococcus uberis* (three strains, FT86, FT126 and FT190). Four other isolates, identified as *Weissella confusa* FT424, *W. hellenica* FT476, *Leuconostoc citreum* FT671 and *Lactobacillus plantarum* FT723, showed antifungal activity in preliminary assays. Among these, the strain with the best antifungal activity was *L. plantarum* FT723, which inhibited the mold *Penicillium expansum* in modified MRS agar (De Man, Rogosa, Sharpe, without acetate) and fermented milk model, whereas no inhibition was observed toward the yeast *Yarrowia lipolytica*. Proteolytic activity was detected in 205 isolates by agar assay and out of these, 123 more proteolytic isolates were submitted to confirmatory test of activity by SDS-PAGE. *Enterococcus faecalis* (strains FT132 and FT522) and *Lactobacillus paracasei* FT700 were confirmed as proteolytic strains by SDS-PAGE and bands indicating digestion of caseins and whey proteins (β-lactoglobulin and α-lactalbumin) were detected. *E. faecalis* FT132, along with *L. paracasei* FT700, were selected for further studies on proteolytic activity using milk proteins as substrates in different

conditions and sequential analyses by SDS-PAGE and high-performance liquid chromatography (HPLC). Both *E. faecalis* FT132 and *L. paracasei* FT700 showed proteolytic activities at pH 6.5, in the range of 37 to 42 °C, due to production of metalloproteases. Next, to evaluate the possible biological activities of the peptides derived from the action of *E. faecalis* FT132 and *L. paracasei* FT700 on milk proteins, the supernatant of fermented milk produced by these strains were cleaned-up with a C_8 cartridge and freeze-dried. Fermented milk supernatants produced by these strains were added to cell cultures (monocytes and macrophages) to evaluate cytotoxicity, associated death mechanisms, immunomodulatory properties (differentiation of monocytes into macrophages) and TNF-α (Tumor necrosis factor) production. The fermented milk supernatants were toxic to monocytes after 72 h of exposure at 10 mg/mL by apoptosis mechanism. Below cytotoxic concentrations, both cell-free supernatants of fermented milk produced by *E. faecalis* FT132 and *L. paracasei* FT700 stimulated the differentiation of monocytes into macrophages (increased expression of CD71 marker). This immune stimulation was not inflammatory since low production of TNF-α was detected. LAB may also present health benefits by acting as probiotics. However, for the selection of probiotics, absence of virulence traits must be proven. Lactobacilli usually have generally recognized as safe (GRAS) status, contrary to enterococci. In this study, it was detected that *E. faecalis* FT132 harbored three virulence genes *asa*1, *ace* and *gel*E, and that it was resistant to erythromycin and tetracycline, indicating that it was not advisable to be added to food products. However, *L. paracasei* FT700 would be a potential probiotic candidate, as well as the bacteriocinogenic strain previously described *L. paraplantarum* FT259. These two strains were tested for probiotic potential by survival in acidified culture media (pH 2.0, 2.5 and 3.5), *in vitro* tolerance to bile salts, viability in simulated gastric juice and antibiotic susceptibility. In addition, the antimicrobial peptide produced by *L. paraplantarum* FT259 was partially purified in column filled with XAD-16 resin, followed by a C_{18} cartridge solid phase extraction, and analyzed by SDS-PAGE. Polymerase chain reactions with primers for plantaricin NC8, plantaricin S and plantaricin W structural genes followed by DNA sequencing of amplicons were carried out to screen for genetic determinants for bacteriocin production. The results showed *L. paraplantarum* FT259 tolerated exposure to pH 3.5, and bile salts (0.3%) for up to 180 minutes, but population of cells exposed to pH 2.0 and 2.5 for 90 minutes were dropped to less than 2 log CFU/mL (detection limit of the method). In experiments with simulated gastric juice, viable cells of *L. paraplantarum* FT259 decreased from 8.6 log CFU/mL to 4.4 log CFU/mL after 180 minutes. Otherwise, *L. paracasei* FT700 survived well in almost all conditions. After 180 minutes in pH 2.0 and simulated gastric juice, bacterial population decreased 4 and 3 log CFU/mL, respectively. It was also demonstrated that *L. paraplantarum* FT259 and *L. paracasei* FT700 were susceptible to the majority the antibiotics tested. SDS-PAGE analysis indicated that the partially purified bacteriocin presented a molecular mass around 3,900 Da and by PCR combined with DNA sequencing it was detected the plantaricin NC8 gene. Overall, these results indicated that both strains have potential probiotic traits. Moreover, the production of bacteriocins (*L. paraplantarum* FT259) and proteolytic activity (*L. paracasei* FT700) may be interesting in food technology application. In conclusion, the LAB obtained in this study may be useful in the food industry to the production of new dairy products with prolonged shelf-life and increased digestibility, or even for the production of immunomodulatory peptides commercialized as partially purified formulas.

Keywords: lactic acid bacteria, antimicrobials, proteolysis, milk, cheese.

RESUMO

TULINI, F. L. **Isolamento de bactérias láticas de leite e queijo com potencial para bioconservação de alimentos e utilização no aumento da digestibilidade de proteínas do lactosoro**. 2014. 182 f. Tese (Doutorado). Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto (Brasil), e École Doctorale Santé, Information-Communication, Mathematiques, Matières – Université de Brest, Brest (França), 2014.

As bactérias láticas (BAL) têm sido utilizadas pela humanidade há séculos devido às suas propriedades tecnológicas e potencial para conferir características sensoriais agradáveis aos alimentos. Além disso, é cada vez maior a demanda por alimentos de qualidade. Neste contexto, BAL tem grande potencial para serem utilizadas na produção de alimentos. Uma das principais características destas bactérias é a produção de substâncias que inibem a multiplicação de agentes patogênicos e micro-organismos deteriorantes em alimentos, tais como ácidos orgânicos, H_2O_2 e bacteriocinas. Estas últimas são um peptídeos antimicrobianos produzido via ribossomo por algumas bactérias e possuem pequeno espectro inibição. As bacteriocinas podem reduzir a multiplicação de bactérias-alvo, aumentando a segurança alimentar e a vida de prateleira de alimentos. Essas substâncias inibitórias produzidas por BAL podem também inibir fungos, que são em grande parte responsáveis pela deterioração dos alimentos. Outra importante propriedade das BAL é capacidade de modificar as proteínas do leite durante o processo de fermentação. Isto é importante para indivíduos alérgicos devido à alteração de potencial alergênico das proteínas do leite, e também é importante para a produção de peptídeos bioativos. Recentemente, resultados demonstraram que os peptídeos obtidos a partir da hidrólise de proteínas do leite podem ter diferentes atividades biológicas, tais como anti-hipertensiva, antioxidante, antimicrobiana e imunomodulatória. Em resumo, BAL apresentam potencial para a produção de alimentos de alta qualidade, o que estimula a busca de novas linhagens com propriedades tecnológicas de interesse. Assim, no presente estudo, BAL com atividade antimicrobiana e / ou proteolítica foram isoladas de leite e queijo de vaca, búfala e cabra, obtidos na região sudeste do Brasil. A partir de 156 amostras de leite e queijo, foram obtidos 815 isolados em ágar seletivo para BAL. A maioria eram cocos ou bacilos Gram-positivos, e não produziam a enzima catalase. Culturas puras destes novos isolados foram avaliadas quanto a atividade antimicrobiana por testes de antagonismo em ágar (*spot-on-the-lawn*), e quanto a atividade proteolítica sobre proteínas do leite pelo cultivo em placas de ágar BHI (*Brain Heart Infusion* suplementado com leite desnatado). Os isolados com maior atividade proteolítica também foram testados pelo cultivo em leite desnatado seguido de análise do leite fermentado por eletroforese em gel de poliacrilamida com dodecil sulfato de sódio (*sodium dodecyl sulfate polyacrylamide gel electrophoresis* SDS-PAGE). Entre os 815 isolados testados, quatro deles foram identificados como produtores de bacteriocinas, *Lactobacillus paraplantarum* FT259 (uma linhagem) e *Streptococcus uberis* (três linhagens, FT86, FT126 e FT190), enquanto quatro outros identificados como *Weissella confusa* FT424, *W. hellenica* FT476, *Leuconostoc citreum* FT671 e *Lactobacillus plantarum* FT723, os quais apresentaram atividade antifúngica em ensaios preliminares. Análises complementares mostraram que a linhagem com maior atividade antifúngica foi *L. plantarum* FT723, o qual inibiu *Penicillium expansum* em ágar MRS modificado (De Man, Rogosa, Sharpe, sem acetato) e em

modelo de leite fermentado. No entanto, nenhuma inibição foi observada contra *Yarrowia lipolytica*. A atividade proteolítica foi detectada em 205 isolados por testes em ágar, sendo que 123 isolados com intensa atividade proteolítica foram submetidos a confirmação da atividade por SDS-PAGE. As atividades proteolíticas de três isolados identificados como *Enterococcus faecalis* (FT132 e FT522) e *Lactobacillus paracasei* FT700 foram confirmadas por SDS-PAGE, como visualizado pela digestão de caseínas e proteínas de soro de leite (β-lactoglobulina e α-lactalbumina). No entanto, devido à alta semelhança entre as linhagens, apenas *E. faecalis* FT132 (juntamente com *L. paracasei* FT700) foram selecionados para os próximos estudos utilizando proteínas do leite como substratos em diferentes condições, seguidas de análises por SDS-PAGE e cromatografia líquida de alta eficiência (HPLC, *high-performance liquid chromatography*). Ambos *E. faecalis* FT132 e *L. paracasei* FT700 apresentaram atividades proteolíticas em pH 6,5, entre 37 e 42 °C. A atividade proteolítica das linhagens foi devida à presença de metaloproteases. Em seguida, para avaliar as possíveis atividades biológicas dos peptídeos derivados da atividade proteolítica de *E. faecalis* FT132 e *L. paracasei* FT700 em proteínas do leite, o sobrenadante de leite fermentado produzido por estas linhagens foi purificado em cartucho C_8 e liofilizado. Desse modo, os sobrenadantes de leite fermentado produzidos por estas linhagens foram adicionados às culturas de células (monócitos e macrófagos) para avaliar a sua citotoxicidade, os mecanismos de morte celular, propriedades imunomoduladoras (diferenciação de monócitos em macrófagos) e quantificação de TNF-α (do inglês *tumor necrosis factor*). Os sobrenadantes apresentaram toxicidade após 72 h de exposição a 10 mg/mL por apoptose. Abaixo das concentrações citotóxicas, ambos os sobrenadantes de leite fermentado estimularam a diferenciação de monócitos em macrófagos, como observado pelo aumento da expressão do marcador CD71. Esta estimulação imune não foi inflamatória visto que houve pouca produção de TNF-α. BAL também podem contribuir para a saúde dos consumidores quando utilizadas como probióticos. No entanto, algumas características das linhagens devem ser verificadas antes de serem utilizadas como probióticos, especialmente com relação aos fatores de virulência. Lactobacilos geralmente possuem um status GRAS (do inglês *generally recognized as safe*), ao contrário dos enterococos. Neste estudo, foi demonstrado que *E. faecalis* FT132 possuía três genes de virulência, *asa*1, *ace* e *gel*E, e que era resistente à eritromicina e à tetraciclina, indicando que esta linhagem não pode ser adicionada em alimentos. No entanto, *L. paracasei* FT700 seria um potencial candidato para ser utilizada como probiótico, bem como a linhagem bacteriocinogênica descrita anteriormente, *L. paraplantarum* FT259. As linhagens foram testadas quanto à sobrevivência em meio ácido (pH 2,0, 2,5 e 3,5), tolerância *in vitro* aos sais biliares, viabilidade em suco gástrico sintético e sensibilidade a antibióticos. Além disso, o peptídeo antimicrobiano produzido por *L. paraplantarum* FT259 foi parcialmente purificado em coluna preenchida com resina XAD-16, seguido de extração em fase sólida com cartucho de C_{18}, e analisados por SDS-PAGE. Reações em cadeia da polimerase (PCR, do inglês *polymerase chain reaction*) com *primers* para os genes estruturais da plantaricina NC8, plantaricina S e plantaricina W, seguidas de sequenciamento de DNA, foram realizados para detectar genes responsáveis pela produção de bacteriocinas. Os resultados mostraram que *L. paraplantarum* FT259 foi resistente ao pH 3,5 e a 0,3% de sais biliares por até 180 minutos, mas a população bacteriana em pH 2,0 e 2,5 após 90 minutos estava abaixo do limite de detecção do método (2 log UFC/mL). Em testes utilizando suco gástrico sintético, a população de *L. paraplantarum* FT259 reduziu de 8,6 log UFC/

mL para 4,4 log UFC/mL após 180 minutos. Por outro lado, *L. paracasei* FT700 sobreviveu bem em quase todas as condições. Depois de 180 minutos em pH 2,0 e em suco gástrico sintético, a população bacteriana reduziu 4 e 3 log UFC/mL, respectivamente. Também foi demonstrado que *L. paraplantarum* FT259 e *L. paracasei* FT700 foram sensíveis à maioria dos antibióticos testados. A análise de SDS-PAGE indicou que a bacteriocina parcialmente purificada apresentava uma massa molecular de aproximadamente 3900 Da, e o sequenciamento de DNA do produto de amplificação obtido por PCR mostrou a presença do gene que codifica a produção da plantaricina NC8. De modo geral, os resultados indicaram que ambas as linhagens possuem potencial probiótico. Além disso, a produção de bacteriocinas (*L. paraplantarum* FT259) e a atividade proteolítica (*L. paracasei* FT700) podem ser características interessantes para aplicações em alimentos. As BAL obtidas neste estudo podem ser úteis na indústria de alimentos para a produção de novos produtos lácteos com maior vida de prateleira e aumento da digestibilidade de proteínas do leite, assim como para a produção de peptídeos bioativos comercializados como fórmulas parcialmente purificadas.

Palavras-chave: bactérias láticas, antimicrobianos, proteólise, leite, queijo.

RÉSUMÉ

TULINI, F. L. **Isolement et caractérisation de bactéries lactiques de produits laitiers brésiliens en vue de leur utilisation potentielle en biopréservation ou pour l'amélioration de la digestibilité des protéines du lactosérum.** 2014. 182 p. Thèse de Doctorat. Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto (Brésil), et l'École Doctorale Santé, Information-Communication, Mathematiques, Matières – Université de Bretagne Occidentale, Brest (France), 2014.

Les bactéries lactiques (BAL) ont été utilisées par l'humanité depuis des siècles en raison de leurs propriétés technologiques et de leur capacité à améliorer les propriétés organoleptiques des aliments. En outre, la demande des consommateurs pour des produits laitiers sains et dépourvus de conservateurs chimiques est de plus en plus grande.Dans ce contexte, l'utilisation de bactéries lactiques utilisées depuis des siècles pour la fermentation des produits laitiers semble pertinente pour améliorer la digestibilité et/ou prolonger la durée de vie des produits laitiers fermentés. L'une des principales propriétés de ces bactéries est la production de substances antimicrobiennes telles que des bactériocines, des peptides ou des composés non protéiques de faible poids moléculaire (acides organiques, H_2O_2, et ainsi de suite) qui inhibent la croissance des pathogènes alimentaires et des micro-organismes d'altération. Les bactériocines sont des peptides antimicrobiens ribosomiques produits entre autre par certaines bactéries lactiques et qui possèdent un spectre d'inhibition étroit ciblant généralement les bactéries à Gram-positif. Les substances inhibitrices produites par BAL peuvent également inhiber les champignons qui sont en grande partie responsables pour la détérioration des aliments. Un autre propriété importante de BAL est de modifier les protéines du lait au cours du processus de fermentation. Ceci est important pour les personnes allergiques, en raison de la modification du potentiel allergénique de protéines du lait, mais également important pour la production de peptides bioactifs. Récentes découvertes ont montré que des peptides dérivés de l'hydrolyse des protéines du lait peuvent avoir des activités biologiques, tels que des activités antihypertensives, antioxydantes, antimicrobiennes et immunomodulatrices. En résumé, les BAL sont des outils potentiels pour la production d'aliments de haute qualité, ce qui stimule la recherche de nouvelles souches ayant des propriétés biologiques intéressantes. Ainsi, l'objectif de cette étude était d'isoler et de cribler de nouvelles souches de BAL possédant des activités antimicrobienne et / où protéolytique. Des souches de BAL ont ainsi été isolées de lait de vache, de buffle et de chèvre ainsi que des fromages provenant du sud-est du Brésil. À partir de 156 échantillons de lait et de fromages, 815 isolats ont été obtenus sur des géloses sélectives pour les BAL. La majorité d'entre elles était des coques ou des bacilles à Gram-positif et négatif pour la production de catalase. La présence d'activités antimicrobiennes a été évaluée sur des cultures pures de ces nouveaux isolats par des tests d'antagonisme sur géloses (essai *spot-on-the-lawn*), et leurs activités protéolytiques on été évaluées par culture sur géloses BHI (*Brian Heart Infusion*) additionnée de lait écrémé. Les isolats les plus protéolytiques ont été testés par culture dans du lait écrémé suivie d'une électrophorèse sur gel de polyacrylamide en condition dénaturante (*sodium dodecyl sulfate polyacrylamide gel electrophoresis* SDS-PAGE). Parmi les 815 isolats testés, quatre d'entre eux, identifiés comme *Lactobacillus paraplantarum* FT 259 et *Streptococcus uberis* (FT86, FT126 et FT190)

étaient producteurs de bactériocines, tandis que quatre autres, identifiés comme *Weissella confusa* FT424, *Weissella hellenica* FT476, *Leuconostocs citreum* FT671 et *Lactobacillus plantarum* FT723 ont montré une activité antifongique lors d'essais préliminaires. Des analyses complémentaires ont montré que la souche la plus antifongique était *L. plantarum* FT723 qui inhibait *Penicillium expansum* sur gélose MRS modifiée (de Man, Rogosa, Sharpe, sans acétate) et dans un modèle de lait fermenté. Des activités protéolytiques ont été détectées dans 205 isolats lors des tests sur géloses supplémentées en lait écrémé. Les activités protéolytiques des 123 isolats présentant les activités les plus fortes ont été confirmées en faisant migrer les surnageants de laits fermentés sur SDS-PAGE. Les capacités protéolytiques des trois isolats les plus protéolytiques *Enterococcus faecalis* (FT132 et FT522) et *Lactobacillus paracasei* FT700 ont également été testées sur des caséines et sur deux protéines du lactosérum (β-lactoglobuline et d'α-lactalbumine), les produits de dégradation ont été visualisés par SDS-PAGE. En raison de la grande similitude entre les deux souches d'*Enterococcus*, seul *E. faecalis* FT132 et *L. paracasei* FT700 ont été sélectionnés pour des études plus poussées sur leurs activités protéolytiques en utilisant des protéines du lait comme substrats lors d'incubations dans différentes conditions suivies de l'analyse des produits d'hydrolyse par SDS-PAGE et par chromatographie liquide haute performance (*high performance liquid chromatography*, HPLC). Tant *E. faecalis* FT132 que *L. paracasei* FT700 ont montré des activités protéolytiques à un pH de 6,5, à des températures comprises entre 37 à 42 ºC. Leurs activités protéolytiques étaient dues à des métallo-protéases. Pour évaluer les activités biologiques possibles des peptides dérivés de l'action d'*E. faecalis* FT132 et *L. paracasei* FT700 sur les protéines du lait, les surnageants des laits fermentés par ces souches ont été purifiés sur colonnes C_8 puis lyophilisés. Ces lyophilisats ont ensuite été repris dans du RPMI (*Roswell Park Memorial Institute* medium) et ajoutés à différentes cultures primaires (monocytes et macrophages) pour évaluer leur cytotoxicité, les mécanismes de la mort celullaire qui y étaient associés, ainsi que leurs propriétés immunomodulatrices (différenciation des monocytes en macrophages et quantification de TNF-α). Les peptides générés par les deux souches testées étaient toxiques (favorisant l'apoptose des cellules) après 72 h d'exposition à une concentration de 10 mg/mL. Au-dessous de ces concentrations cytotoxiques, les deux surnageants de laits fermentés produits par *E. faecalis* FT132 et *L. paracasei* FT700 ont stimulé la différenciation des monocytes en macrophages, comme observé par l'expression accrue du marqueur CD71. Cette stimulation du système immunitaire n'était pas inflammatoire en raison de la faible production de TNF-α. Certaines BAL peuvent contribuer à la santé des consommateurs et sont utilisées comme probiotiques. Cependant, les effets biologiques des souches ainsi que leur innocuité doivent être vérifiés avant leur utilisation comme probiotiques. Contrairement aux entérocoques, plusieurs espèces de lactobacilles sont reconnues comme GRAS (*Generally Recognized as Safe*). Dans cette étude, il a été montré que la souche *E. faecalis* FT132 hébergeait trois gènes de virulence *asa*1, *as* et *gel*E, et qu'elle était résistante à l'érythromycine et à la tétracycline, ce qui signifie que cette souche ne peut pas être ajoutée à des produits alimentaires. Cependant, *L. paracasei* FT700 pourrait d'avéré un candidat potentiel pour être utilisée en tant que probiotique, ainsi que *L. paraplantarum* FT259, la souche bacteriocinogénique décrite précédemment. Afin d'évaluer leur capacité de survie dans le tractus digestif en vu d'une éventuelle utilisation comme probiotiques, les souches ont été incubées dans des milieux de culture acidifiés (pH 2,0, 2,5 et 3,5), dans des sels biliaires, dans des conditions simulant les conditions

gastriques et leur capacité de survie a été testée ainsi que leur sensibilité à différents antibiotiques. En outre, la bactériocine produite par *L. paraplantarum* FT259 a été partiellement purifiée sur colonne remplie de résine XAD-16, suivie d'une extraction en phase solide sur colonne C_{18}, suivie d'une analyse par SDS-PAGE. Des PCR avec différentes amorces ciblant la plantaricine NC8, la plantaricine S et la plantaricine W ont été effectuées suivie du séquençage des amplicons afin d'idéntifier des gènes pour la production de bactériocines. Les résultats ont montré que *L. paraplantarum* FT259 tolérait des expositions à un pH de 3,5, et à une concentration en sels biliaires de 0,3% pendant une durée maximum de 180 minutes. En revanche, il n'était plus possible de détecter de cellules viables (limite de détection de la méthode : 100 UFC / mL) après une exposition à des pH de 2,0 et de 2,5 pendant 90 minutes. Lors des expériences simulant les conditions gastriques, le nombre de cellules viables de *L. paraplantarum* FT259 a diminué de 8,6 log UFC/mL à 4,4 log UFC/mL après 180 minutes d'incubation. Les résultats obtenus pour la souche *L. paracasei* FT700 ont montré que celle-ci a survécut à presque toutes les conditions. Après 180 minutes dans un pH de 2,0 et dans le jus gastrique simulé, la population bactériennes ont respectivement diminué de 4 et de 3 log UFC/mL. Il a également été démontré que *L. paraplantarum* FT259 et *L. paracasei* FT700 étaient sensibles à la majorité des antibiotiques testés. L'analyse par SDS-PAGE a indiqué que la bactériocine partiellement purifiée présentait une masse moléculaire d'environ 3900 Da et que le séquençage des amplicons d'ADN suite aux PCR a montré que la souche possédait le gène de la plantaricine NC8. L'ensemble de ces résultats indique que les deux souches de lactobacilles isolées lors de cette étude (*L. paraplantarum* FT259 et *L. paracasei* FT700) possèdent des caractéristiques pouvant leur conférer un intérêt d'utilisation en tant queprobiotiques. La production de bactériocines (*L. paraplantarum* FT259), et l'activité protéolytique (*L. paracasei* FT700) pourraient également être des caractéristiques intéressantes pour des applications alimentaires. En conclusion, les BAL obtenues dans cette étude pourraient être utiles dans l'industrie alimentaire pour la production de nouveaux produits laitiers à durée de conservation prolongée et une digestibilité accrue, ou encore pour la production de peptides bioactifs.

Mots-clés: bactéries lactiques, des agents antimicrobiens, protéolyse, lait, fromage.

RESUMEN

TULINI, F. L. **Aislamiento de bacterias del ácido láctico en la leche y en el queso con potencial para biopreservation en alimentos y uso para aumentar la digestibilidad de las proteínas de suero de leche.** 2014. 182 p. Tesis (Doctorado). Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto (Brasil), y École Doctorale Santé, Information-Communication, Mathematiques, Matières – Université de Brest, Brest (Francia), 2014.

Las bacterias lácticas (BAL) han sido utilizadas por la humanidad desde hace siglos debido a sus propiedades tecnológicas y debido a su capacidad de mejorar las propiedades organolépticas de los alimentos. Además, la demanda de los consumidores para productos lácteos sanos y privados de conservadores químicos es cada vez más grande. En este contexto, la utilización de bacterias lácticas utilizadas desde hace siglos para la fermentación de los productos lácteos parece pertinente para mejorar la digestibilidad y/o prolongar la vida útil de los productos lácteos fermentados. Una de las principales propiedades de estas bacterias es la producción de sustancias antimicrobianas tales como bacteriocinas, péptidos o compuestos no proteicos de bajo peso molecular (ácidos orgánicos, H_2O_2 y otros) que inhiben el crecimiento de patógenos alimentarios y microorganismos causantes de deterioro. Las bacteriocinas son péptidos antimicrobianos producidos por ciertas bacterias lácticas y quienes poseen un espectro estrecho de inhibición que generalmente se dirige a las bacterias Gram-positivas. Las sustancias inhibitorias producidas por BAL también pueden inhibir por los hongos, que son en gran parte responsables para el deterioro de los alimentos. Una otra propiedad importante de BAL es modificar las proteínas de la leche en el curso del proceso de fermentación. Esto es importante para las personas alérgicas, debido a la modificación del potencial alergénico de proteínas de la leche, y también por la producción de péptidos bioactivos. Descubrimientos recientes mostraron que péptidos derivados de la hidrólisis de las proteínas de la leche podrían tener actividades biológicas, tales como actividades antihipertensivas, antioxidantes, antimicrobianas e inmunomoduladoras. En resumen, BAL son herramientas potenciales para la producción de alimentos de alta calidad, lo que estimula la búsqueda de nuevas cepas que tienen propiedades biológicas interesantes. Así, el objetivo de este estudio era aislar y caracterizar nuevas cepas de BAL que poseían actividades antimicrobianas y / o eran proteolíticas. Cepas de BAL han sido aisladas de leche y queso de vaca, búfala y cabra, obtenidos del sudeste de Brasil. A partir de 156 muestras de leche y de quesos, 815 cepas han sido obtenidas sobre agar selectivo para BAL. La mayoría de ellas eran cocos o bacilos a Gram-positivos y negativo para la producción de catalasa. La presencia de actividades antimicrobianas han sido evaluadas sobre culturas puras de estas nuevas cepas por pruebas de antagonismo sobre agar (ensayo *spot-on-the-lwan*), y sus actividades proteolíticas han sido evaluadas por cultura sobre agar BHI (*Brian Heart Infusion*) añadido de la leche desnatada. Las cepas más proteolíticas han sido sometidas a un test por cultura en leche desnatada seguida por una electroforesis en gel de poliacrilamida con dodecilsulfato sódico (*sodium dodecyl sulfate polyacrylamide gel electrophoresis*, SDS-PAGE). Entre las 815 cepas aisladas y evaluadas, cuatro de ellas, identificadas como *Lactobacillus paraplantarum* FT 259 y *Streptococcus uberis* (FT86, FT126 y FT190) eran productoras de bacteriocinas, mientras que cuatro otras, identificadas como *Weissella confusa* FT424, *Weissella hellenica* FT476, *Leuconostoc citreum*

FT671 y *Lactobacillus plantarum* FT723 mostraron una actividad antifúngica en ensayos preliminares. Análisis complementarios mostraron que la cepa más antifúngica era *L. plantarum* FT723, que inhibía *Penicillium expansum* sobre agar modificado (de Man, Rogosa, Sharpe, sin acetato) y en un modelo de leche fermentada. Actividades proteolíticas han sido detectadas en 205 cepas en el agar añadido de la leche desnatada. Las actividades proteolíticas de las 123 cepas presentando las actividades más fuertes han sido confirmadas haciendo emigrar los sobrenadantes de leches fermentados por SDS-PAGE. Las capacidades proteolíticas de las tres cepas más proteolíticas, *Enterococcus faecalis* (FT132 y FT522) y *Lactobacillus paracasei* FT700, también han sido evaluadas sobre caseínas y sobre proteínas del suero de leche (β-lactoglobulina y α-lactalbumina), y los productos de degradación han sido analizados por SDS-PAGE. Debido a la gran similitud entre ambas cepas de *Enterococcus*, solamente *E. faecalis* FT132 y *L. paracasei* FT700 han sido seleccionados para los estudios siguientes. La actividad proteolítica utilizando proteínas de la leche como substratos fue realizada en diferentes condiciones de incubaciones, seguida del análisis de los productos de hidrólisis por SDS-PAGE y por cromatografía líquida alta eficiencia (*high performance liquid chromatography*, HPLC). *E. faecalis* FT132 y *L. paracasei* FT700 mostraron actividades proteolíticas a un pH de 6,5, y a temperaturas comprendidas entre 37 a 42 °C. Sus actividades proteolíticas fueron debidas a metaloproteasas. Para evaluar las posibles actividades biológicas de los péptidos derivados de la acción de *E. faecalis* FT132 y *L. paracasei* FT700 sobre las proteínas de la leche, los sobrenadantes de las leches fermentadas por estas cepas han sido purificados por columna C_8 y liofilizados. Estos productos han sido resuspendidos en el RPMI (*Roswell Park Memorial Institute* medium) y añadidos a diferentes culturas primarias (monocitos y macrófagos) para evaluar su citoxicidad, los mecanismos de la muerte celular, así como sus propiedades inmunomoduladoras (diferenciación de los monocitos en macrófagos y cuantificación de TNF-α). Los péptidos generados por ambas cepas eran tóxicos (favoreciendo el apoptosis de las células) después de 72 horas de exposición a concentración de 10 mg/mL. Abajo de estas concentraciones citotóxicas, los dos sobrenadantes de las leches fermentadas producidas por *E. faecalis* FT132 y *L. paracasei* FT700 estimularon la diferenciación del monocitos en macrófagos, como observado por la expresión aumentada del marcador CD71. Esta estimulación del sistema inmunitario no era inflamatoria debido a la pequeña producción de TNF-α. BAL pueden contribuir a la salud de los consumidores y son utilizadas como probióticos. Sin embargo, los efectos biológicos de las cepas así como su inocuidad deben ser verificados antes de su utilización como probióticos. Contrariamente al enterococos, varias especies de lactobacilos son reconocidas como GRAS (*Generally Recognized As Safe*). En este estudio, ha sido mostrado que la cepa *E. faecalis* FT132 albergaba tres genes de virulencia, el *asa*1, *as* y *gel*E, y que era resistente al eritromicina y al tetraciclina, lo que significa que esta cepa no puede ser añadida a productos alimentarios. Sin embargo, *L. paracasei* FT700 podría ser un candidato potencial para ser utilizado como probiótico, así como *L. paraplantarum* FT259, la cepa productora de bacteriocina descrita anteriormente. Con el objetivo de evaluar su capacidad de supervivencia en el tracto digestivo por una utilización eventual como probióticos, las cepas han sido incubados en medios de cultura acidificados (pH 2,0, 2,5 y 3,5), en sales biliares, en condiciones que simulan las condiciones gástricas y su sensibilidad a diferentes antibióticos. Además, la bacteriocina producida por *L. paraplantarum* FT259 ha sido purificada parcialmente con una columna llena de resina XAD-16, seguida de una extracción

en fase sólida con columna C_{18} y de un análisis por SDS-PAGE. Para evaluar los genes responsables por la producción de bacteriocinas, fueron realizadas reacciones en cadena de la polimerasa (PCR) con diferentes *primers* que se dirigen al plantaricina NC8, plantaricina S y plantaricina W. Además, ha sido efectuada la secuenciación de amplicones con el objetivo de identificar genes responsables de la producción de bacteriocinas. Los resultados mostraron que *L. paraplantarum* FT259 toleraba exposiciones a un pH de 3,5, y a una concentración en sales biliares del 0,3 % durante una duración máxima de 180 minutos. En cambio, no era posible más detectar las células viables (límite de detección del método: 2 log UFC/mL) después de una exposición a pH de 2,0 y de 2,5 durante 90 minutos. En las condiciones que simulaban las condiciones gástricas, el número de células viables de *L. paraplantarum* FT259 le disminuyó de 8,6 log UFC/mL a 4,4 log UFC/mL después de 180 minutos de incubación. Los resultados obtenidos para la cepa *L. paracasei* FT700 mostraron que ésta cepa sobrevivió casi en todas las condiciones. Después de 180 minutos en un pH de 2,0 y en un zumo gástrico simulado, las poblaciones bacterianas disminuyeron, respectivamente, 4 y 3 log UFC/mL. También ha sido demostrado que *L. paraplantarum* FT259 y *L. paracasei* FT700 eran sensibles a la mayoría de los antibióticos evaluados. El análisis por SDS-PAGE indicó que la bacteriocina parcialmente purificada presentaba una masa molecular de cerca de 3900 Da y que la secuenciación de amplicones obtenidos por PCR mostró la presencia del gene de la plantaricina NC8. El conjunto de estos resultados indica que ambas cepas de lactobacilos aisladas en el momento de este estudio (*L. paraplantarum* FT259 y *L. paracasei* FT700) poseen características que pueden conferirles un interés de utilización como probióticos. La producción de bacteriocinas (*L. paraplantarum* FT259), y la actividad proteolítica (*L. paracasei* FT700) también podrían ser características interesantes para aplicaciones alimentarias. En conclusión, BAL obtenidas en este estudio podrían ser útil en la industria alimentaria para la producción de nuevos productos lácteos de conservación prolongada y una digestibilidad aumentada, o todavía para la producción de péptidos bioactivos.

Palabras clave: bacterias de ácido láctico, agentes antimicrobianos, proteólisis, leche, queso.

FIGURES

TABLES

LIST OF ABBREVIATIONS AND ACRONYMS

AAD	Antibiotic-associated diarrhea
AMS	Acidified milk supernatant
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
AU	Arbitrary unit
BHI	Brain Heart Infusion
ca.	*Circa*
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
CMA	Cow milk allergy
d	Density
DNA	Deoxyribonucleic acid
e.g.	*Exempli gratia*
EDTA	Ethylenediamine tetraacetic acid
FAO	Food and Agriculture Organization of the United Nations
FMS	Fermented milk supernatant
GE	General Electric
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRAS	Generally recognized as safe
GTI	Gastrointestinal tract
HPLC	High-performance liquid chromatography
i.e.	*Id est*
IAL	Instituto Adolfo Lutz
IFN-γ	Interferon gama
IL	Interleukin
LAB	Lactic acid bacteria
MCA	Milk citrate agar
MRS	De Man, Rogosa, Sharpe
NSLAB	Non-starter lactic acid bacteria
OD_{600}	Optical density at 600 nm
PBMC	Peripheral blood mononucleated cell
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PCR-DGGE	Polymerase chain reaction - denaturing gradient gel electrophoresis
PDA	Potato dextrose agar
PI	Propidium iodide
RFLP	Restriction fragment length polymorphism)
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAE	Tris-acetate-EDTA
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TNF-α	Tumor necrosis factors alpha
UHT	Ultra-high temperature
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
WHO	World Health Organization
YEM	Yeast extract-malt

LIST OF SYMBOLS AND UNITS

%	Percentage
®	Registered sign
°C	Celsius degree
µg	Microgram
µg/mL	Microgram per milliliter
µL	Microliter
µm	Micrometer
µM	Micromolar (µmol/L)
µmol	Micromole
AU	Arbitrary unit
AU/mL	Arbitrary unit per milliliter
bp	Base pairs
CFU/mL	Colony forming unit per milliliter
cm	Centimeter
Da	Dalton
g	Gram
g	Gravitational force
g/L	Gram per liter
h	Hour
Kb	Kilobase
kDa	Kilodalton
L	Liter
log	Logarithm
M	Molar (mol/L)
mA	Milliamper
mg	Milligram
mg/mL	Miligram per milliliter
min	Minute
mL	Milliliter
mL/min	Milliliter per minute
mm	Millimeter
mM	Millimolar (mmol/L)

mmol	Millimole
mol	6.02 x 10^{23} entities
ng	Nanogram
nm	Nanometer
pg	Picogram
pmol	Picomole
™	Trademark
V	Volt
v	Volume
v/v	Volume per volume
w/v	Weight per volume
α	Greek small letter alpha
β	Greek small letter beta
γ	Greek small letter gama

CONTENTS

1. Introduction

1. Introduction

Lactic acid bacteria (LAB) are a phylogenetically diverse group of Gram-positive and non-sporulating bacteria that have in common the metabolism with production of lactic acid from glucose. They include different genera of the order Lactobacillales such as *Streptococcus*, *Lactococcus*, *Enterococcus*, *Pediococcus*, *Leuconostoc*, *Lactobacillus*, *Carnobacterium*, *Aerococcus*, *Tetragenococcus*, *Vagococcus*, *Oenococcus* and *Weissella* (PFEILER; KLAENHAMMER, 2007).

The LAB predominates in the natural microbiota of many food products (*e.g.*, milk, meat, vegetables, and cereals) and is of high economic importance to the food industry due to their use as starter and non-starter LAB (NSLAB) as well as in food preservation or protection against spoilage processes (STILES; HOLZAPFEL, 1997). Also, antagonistic activity of LAB is important for inhibition of foodborne pathogens (DE VUYST; LEROY, 2007). Autochthonous NSLAB are implicated in cheese ripening and generally consist of mesophilic lactobacilli, pediococci, enterococci and leuconostoc (CASEY et al., 2006). Their role in cheese production is not completely understood, in contrast to the well-known starter LAB. Nevertheless, it is known that NSLAB increase the level of free amino acids, peptides and free fatty acids, which contributes to flavor intensity and to the ripening of cheese. (DE ANGELIS et al., 2001).

Nowadays, researchers have different approaches to identify bacteria in food samples, such as the classical culture-dependent method (followed by phenotypic and/or genotypic identification), and also culture-independent methods (*e.g.* polymerase chain reaction with denaturing gradient gel electrophoresis, PCR-DGGE). Thereby, van Hoorde et al. (2008) evaluated the diversity of lactic acid bacteria in two Flemish artisanal Gouda-type cheeses by culture-dependent and the culture–independent method PCR-DGGE. When using culture-dependent methods, they detected *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus rhamnosus* and *Pediococcus pentosaceous*. However, when using PCR-DGGE, those authors found three species (*Enterococcus faecalis*, *Lactobacillus parabuchneri* and *Lactobacillus gallinarum*) that were not detected in culture-dependent methods. Despite culture-independent methods may result in more accuracy concerning the diversity of microbial population, culture-dependents methods are still very useful for the determination of a number of viable

microorganisms from different microbial groups and their isolation for further studies (ALEGRIA et al., 2012)

Dairy products are important sources of new LAB strains with technological properties. In 2010, Nespolo and Brandelli isolated LAB from sheep milk and cheese with proteolytic, lipolytic and antimicrobial activities. Among the isolates, *L. plantarum* and *L. rhamnosus* presented antimicrobial activity against both Gram-positive and Gram-negative bacteria. Similarly, in 2012, Yang et al. isolated 138 LAB strains from cheese and yogurts, and 20% presented antimicrobial activity against bacteria and fungi. Overall, LAB comprise an important group of microorganisms for the production and development of new food products.

1.1 Safety issues about lactic acid bacteria

From immemorial times, LAB are important for the production of fermented foods so they may be recognized as safe (CLEMENTI; AQUILANTI, 2011). However, the possibility of exchanging genes encoding antibiotic resistance has highlighted the importance of a correct safety evaluation when selecting strains for food application (DICKS; BOTES, 2010). There is also a concern whether these bacteria may transfer their resistance genes to the microbiota of the gastrointestinal tract, or even acquire virulence factors from pathogens (VAN REENEN; DICKS, 2011).

Rare cases of infections by LAB have been reported in patients receiving antibiotic treatment or severely immune compromised (VAN REENEN; DICKS, 2011). Recently, Franko et al. (2013) reported an infectious endocarditis and bacteremia by *L. paracasei* in a 77-year-old male consumer of probiotics, after being submitted to a colonoscopy. The patient was recovered after administration of amoxicillin and gentamicin. Moreover, according to those authors, probiotic may have contributed to this case and its use should be discontinued before digestive surgery and colonoscopy. In 2014, Sadowska-Krawczenko et al. reported that *Lactobacillus rhamnosus* GG caused infection in a newborn child. That infant had been treated empirically with antibiotics and the probiotic *L. rhamnosus* GG to avoid antibiotic-associated gastrointestinal complications. Those authors, however, stated that the use of probiotics should not be discouraged, but their use should be carefully evaluated. Similarly, cases of endocarditis have frequently been reported in elderly people where the causative agent was *Lactococcus garvieae*, a fish pathogen related

to different opportunistic infections in humans such as bacteremia, endocarditis, osteomyelitis, liver abscess and peritonitis (NAVAS; HALL; EL BEIJANI, 2013; ORTIZ et al., 2014; RASMUSSEN et al., 2014; WATANABE et al., 2011).

The safety issues for *Enterococcus* spp. remains controversial as isolates of this genus have emerged as opportunistic pathogens for humans (OGIER; SERROR, 2008). In addition, enterococci have been recognized as important hospital-acquired pathogens in recent years, and isolates of *Enterococcus faecium* and *E. faecalis* are the third- to fourth-most prevalent nosocomial pathogen worldwide (WERNER et al., 2013).

Antibiotic resistance is also a concern when selecting LAB for food applications. *L. plantarum*, *Lactobacillus casei*, *Lactobacillus salivarius*, *Lactobacillus leishmannii*, *Lactobacillus acidophilus*, pediococci and *Leuconostoc* spp. carry intrinsic resistance to vancomycin but, usually, they are not resistance to other antibiotics of clinical interest, which encourage their use in food (BERNARDEAU et al., 2008; OGIER et al., 2008). Most of lactococci species are intrinsically resistant to metronidazole, trimethoprim, cefoxitin, gentamicin and kanamycin, but they are susceptible to antibiotics targeting Gram-positive bacteria, broad-spectrum antibiotics, and also to beta-lactams (DEVIRGILIIS; ZINNO; PEROZZI, 2013). Enterococci are intrinsically resistant to cephalosporins and low levels of aminoglycoside and clindamycin (MATHUR; SINGH, 2005). However, when located on mobile genetic elements (plasmids and transposons), antibiotic resistance traits may be transferred to commensal microbiota and pathogenic bacteria, which reinforce the need of a complete evaluation of safety aspects of LAB before its use for human or animal nutrition (KLARE et al., 2007). In this context, the most concerning LAB are the enterococci. In this genus, mobile elements or foreign DNA within a particular genome (chromosomes and plasmids) may account for 38 % and more than 25 % of the total genome of *E. faecium* and *E. faecalis*, respectively (WERNER et al., 2013). Also, enterococcal antibiotic resistance is well documented and there is evidence of infections by vancomycin resistant enterococci caused by strains acquired through the food chain (FISHER; PHILLIPS, 2009). According to Rubinstein and Keynan (2013), enterococcal infection can be caused by vancomycin-susceptible strains, mainly *E. faecalis*, and vancomycin-resistant strains, mainly *E. faecium*.

1.2 Main foodborne bacterial pathogens and spoilage microorganisms in dairy products

Foodborne pathogens represent a real problem for public health throughout the world (BAJPAI et al., 2008). In a recent review done by Gomes, Franco and De Martinis (2013), *Salmonella* spp., *Staphylococcus* spp., *Bacillus cereus*, *Clostridium perfringens*, *Shigella* spp. and *Clostridium botulinum* were presented as the most common foodborne pathogens reported in outbreaks in Brazil. However, according to those authors, for up to 50% of the registered outbreaks the etiological agent may remain undefined. In Europe, the main foodborne pathogens are *Salmonella* and *Campylobacter*, and their incidence may greatly vary according to the country and year (PIRES et al., 2010). Similarly, according to data from the Centers for Disease Control and Prevention (2011), the bacteria causing the most illnesses, hospitalizations, and deaths in the United States of America (USA) are *Campylobacter* spp., *C. perfringens*, *Escherichia coli* (STEC, Shiga toxin-producing *E. coli*) O157, *Listeria monocytogenes*, nonthyphoiddal *Salmonella* and *Staphylococcus aureus*. Among them, *L. monocytogenes* deserves special attention due to the high mortality associated with listeriosis (MILILLO et al., 2012).

L. monocytogenes, *Listeria marthii*, *Listeria innocua*, *Listeria welshimeri*, *Listeria seeligeri*, *Listeria ivanovii*, *Listeria fleischmannii*, *Listeria aquatica*, *Listeria floridensis*, *Listeria rocourtiae*, *Listeria weihenstephanensis*, *Listeria cornellensis*, *Listeria grandensis*, *Listeria riparia* and *Listeria grayi* compose the genus *Listeria*, but only *L. monocytogenes* and *L. grayi* have been generally considered as pathogens (DEN BAKKER et al., 2014; GASANOV; HUGHES; HANSBRO, 2005). *L. monocytogenes* is a nonspore-forming Gram-positive rod, facultative anaerobic, motile due to peritrichous flagella, and tolerates adverse environmental conditions such as low temperature, high concentration of sodium chloride and low pH (GANDHI; CHIKINDAS, 2007). *L. monocytogenes* can easily contaminate products such as pasteurized milk, soft cheeses, meat products, and ready-to-eat food that are stored at refrigeration temperatures and consumed without previous treatment (MARTINEZ; BRAVO; RODRIGUEZ, 2005). This bacterium is ubiquitous in the environment and it is well adapted to both lives in soil and into the cytosol of eukaryotic host cells (FREITAG; PORT; MINER, 2009). When infecting mammalian hosts, *L. monocytogenes* is able to cross the intestinal, feto-placental and blood brain

barriers and disseminate throughout the organism (COSSART; LEBRETON, 2014). Listeriosis is a relatively rare life-threatening disease (fatality rate of 20 - 30%) that mostly affects immunocompromised individuals, elderly, pregnant women and unborn or newly delivered infants (NEWELL et al., 2010). *Listeria monocytogenes* is one of the main pathogens of concern for the dairy industry, along with *S. aureus*, *E. coli*, and *Salmonella* spp. (GÁLVEZ et al., 2008).

Besides foodborne pathogens, the control of spoilage microorganisms in food is also important. In dairy products, *Pseudomonas* and *Bacillus* may contribute to food deterioration (FRANCO; LANDGRAF, 2005). *Pseudomonas* is a Gram-negative, aerobic, rod-shaped bacterium that grows rapidly in low temperatures and dominates the spoilage microbiota of proteinaceous foods such as milk, meat, poultry and fish, stored aerobically at refrigeration temperatures (MCMEEKIN; ROSS, 1996). In milk, *Pseudomonas* spp. have strong proteolytic and lipolytic activities (SØRHAUG; STEPANIAK, 1997). However, this microorganism usually contaminates pasteurized milk in post-process stages, and milk may also spoil due to psychotropic heat resistant spore of *Bacillus* (GRAM et al., 2002).

Some members of LAB may also be responsible for the spoilage of vacuum packaged refrigerated processed meats and some dairy products (HOLZAPFEL, 1992). In milk, *Lactobacillus* spp. and *Lactococcus lactis* may increase viscosity, and *Lactobacillus lactis* var. *maltigenes* may alter milk aroma (FRANCO; LANDGRAF, 2005).

Microbial contamination of food by fungi (*e.g. Aspergillus*, *Fusarium* and *Penicillium*) represents a real problem to food safety due to the production of mycotoxins, a toxic metabolite produced by certain fungi that may be carcinogenic, immunotoxic, teratogenic, neurotoxic, nephrotoxic and hepatotoxic (DALIÉ; DESCHAMPS; RICHARD-FORGET, 2010). In addition, yeasts may also contaminate food products and are recognized as the main factor for food spoilage. The foodborne yeasts *Candida*, *Debaryomyces*, *Kluyveromyces*, *Rhodotorula*, *Trichosporon* and *Yarrowia* are found quite frequent, and the contamination of food by these yeasts results in changes of food texture, colour and flavor (KUNICKA-STYCZYNSKA, 2011). Poor factory hygiene, lack of preservatives, inadequate pasteurizing temperatures and/or use of poor quality raw materials may contribute for the contamination of spoilage yeasts (GORETTI et al., 2009).

1.3 Potential of lactic acid bacteria for use in food biopreservation

The high demand for healthy food with extended shelf-life is a real challenge for food industry. Traditional strategies used to control microbial contamination in food includes drying, freeze-drying, cold storage, modified atmosphere storage and the additives acetic, lactic, propionic, sorbic and benzoic acids (SCHNÜRER; MAGNUSSON, 2005). LAB may help in biopreservation approaches alternatives or combined with physical and chemical methods to control pathogenic bacteria and spoilage microbiota, due to the production of antimicrobials (DALIÉ; DESCHAMPS; RICHARD-FORGET, 2010).

Microbial inhibition exerted by LAB is generally due to production of increased acidity, competition for substrates, production of antimicrobial compounds such as low-weight non-proteinaceous compounds (organic acids, H_2O_2, and others), bacteriocins and/or antifungal compounds (PARENTE; RICCIARDI, 1999).

1.3.1 Bacteriocins

Bacteriocins are antimicrobial peptides synthesized by bacteria via ribosomes and represent a defense strategy (OSCARIZ; PISABARRO, 2001). These compounds represent a potential alternative to chemical food preservatives because they are generally considered as safe, since they are destroyed by proteolytic enzymes in the gastrointestinal tract (ABRIOUEL et al., 2003; GUINANE et al., 2005). Moreover, many bacteriocins can be detected in fermented meat and dairy products as LAB represent a significant population of these food microbiota. This suggests that they have been consumed by humans for many years, reinforcing safety issues. For example, the antimicrobial peptide nisin (available for commercial use) is produced by certain *Lactococcus lactis* strains, and it is approved for use in food in more than 40 countries for *ca*. five decades (CLEVELAND et al., 2001). Formulations containing bacteriocins such as Nisaplin® (nisin) and ALTA® 2351 (pediocin PA-1) have been added to food to extend shelf-life and increase food safety (MILLS et al., 2011a)

Bacteriocins usually have a narrow spectrum of activity, and the bacteriocinogenic strains are immune to their own bacteriocins (CLEVELAND et al., 2001). The widely used classification system of Cotter, Hill and Ross (2005) divides bacteriocins into classes I, II and III, based on a previous system proposed by

Klaenhammer (1994). The class I bacteriocins (lantibiotics) are extensively post-translationally modified, with unusual amino acids such as lanthionine, methyllanthionine, dehydroalanine, and dehydrobutyrine. Many lantibiotics interfere with bacterial cell wall synthesis and promote pore formation (ROSS; VEDERAS, 2011). Class II comprises thermostable bacteriocins with molecular weight lower than 10 kDa and no modified amino acids. These bacteriocins usually act by permeabilizing the target cell membrane and this class is divided into four subgroups: (i) class IIa bacteriocins, such as pediocin, with the amino-acid sequence motif YGNGV and a disulfide bridge near the N-terminus; (ii) class IIb, that comprise two-peptides bacteriocins; (iii) class IIc bacteriocins with N- and C- termini covalently linked (circular bacteriocins); (iv) in the class IId are allocated the remaining bacteriocins that do not fit in the other subgroups. Finally, class III (bacteriolysins) comprises thermolabile proteins with molecular weights greater than 30 kDa that catalyze the hydrolysis of bacterial cell wall (COTTER; HILL; ROSS, 2005). Figure 1.1 illustrates the three mode of action of each bacteriocin group proposed by Cotter, Hill and Ross (2005).

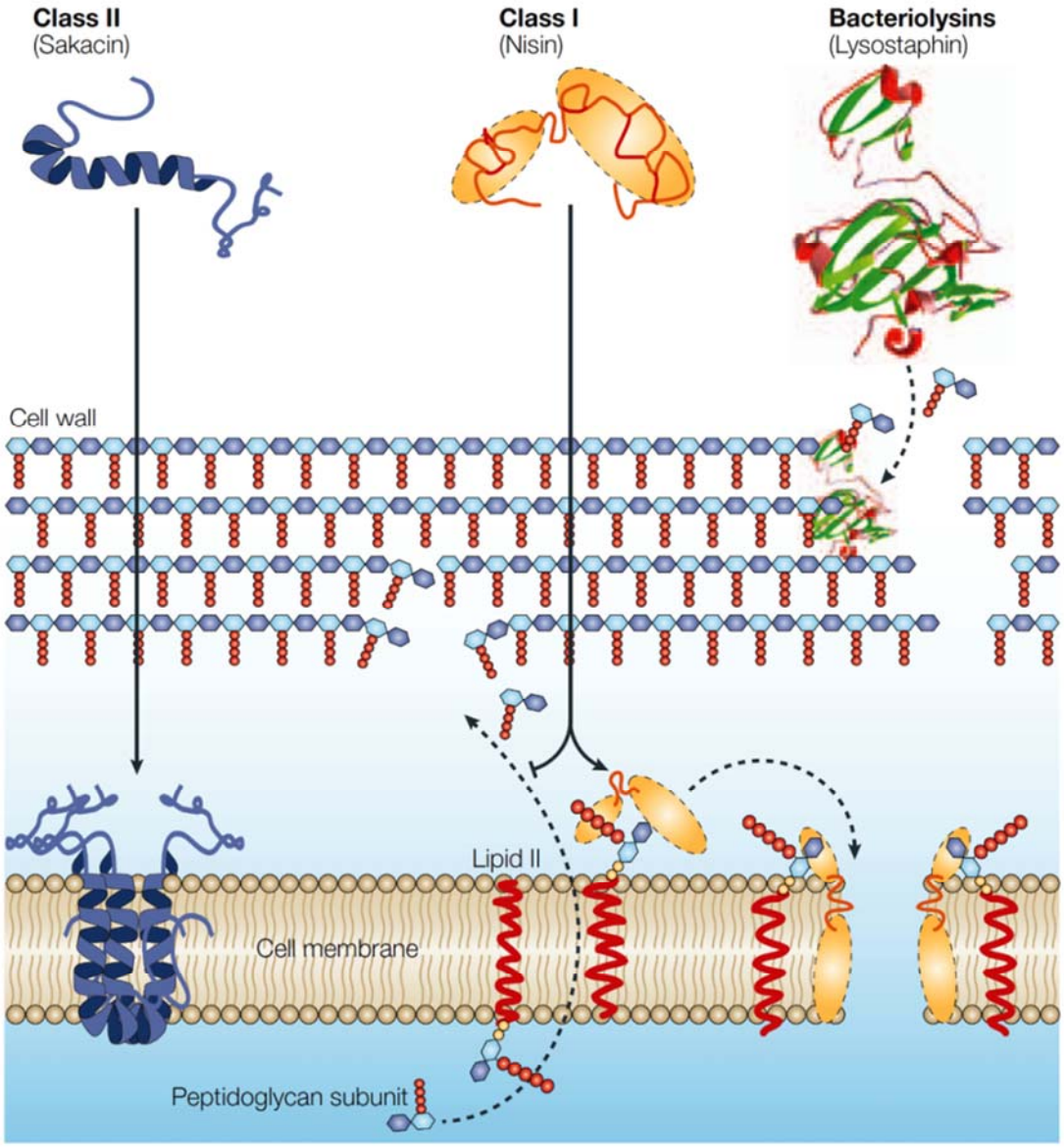


Figure 1.1 Mode of action of bacteriocins from classes I, II and III. In the class I (lantibiotics), the peptides can bind to lipid II (transporter of peptidoglycan subunits from the cytoplasm to the cell wall) and prevent the correct cell wall synthesis, leading to cell death. In addition, lantibiotics can use the lipid II as a tool to initiate the process of membrane insertion and pore formation, also leading to cell death. Bacteriocins allocated in class II have amphiphilic helical structures that allow them to insert into bacterial membrane and cause depolarization, which leads to cell death. In the third class, the bacteriolysins catalyze the hydrolysis of cell wall of Gram-positive bacteria, leading to bacterial lysis and death (COTTER; HILL; ROSS, 2005).

1.3.2 Phenyllactic acid

Phenyllactic acid may be produced by LAB and has broad antibacterial spectrum, antifungal action and it is not toxic to animal and human cells (CROWLEY; MAHONY; VAN SINDEREN, 2013).

Lavermicocca, Valerio and Visconti (2003) evaluated the effects of phenyllactic acid on the growth of 23 strains of *Aspergillus*, *Penicillium* and *Fusarium* isolated from bakery products. Those authors found that less than 7.5 mg/mL of phenyllactic acid was required to inhibit 90% of the growth of all strains, and less than 10 mg/mL was necessary for fungicidal activity in 19 strains. Ndagano et al. (2011) investigated the production of phenyllactic acid by LAB grown in MRS broth without acetate. Those authors detected 0.065 mg/mL (0.48 mM), 0.014 mg/mL (0.10 mM) and 0.018 mg/mL (0.13 mM) of phenyllactic acid, respectively, in the cell free supernatant of the LAB *L. plantarum* VE56, *Weissella cibaria* FMF4B13 and *Weisella paramesenteroides* LC11. Similarly, Cortés-Zavaleta et al. (2014) evaluated the production of phenyllactic acid by 13 lactobacilli with known antifungal activity. According to those authors, the production of phenyllactic acid by these lactobacilli ranged from 0.003 mg/mL (0.021 mM) to 0.037 mg/mL (0.275 mM). These concentrations of phenyllactic acid are lower than the minimal inhibitory concentration (MIC) reported by Lavermicocca et al. (2003), and antimicrobial activity detected suggests there is a synergistic effect with organic acids (CORTÉS-ZAVALETA et al., 2014; NDAGANO et al., 2011).

Mu et al. (2009) evaluated the production of phenyllactic acid by *Lactobacillus* sp. SK007 using batch and fed-batch fermentation. Those authors suggested phenylpyruvic acid is an important substrate to improve the phenyllactic acid production.

1.3.3 Reuterin and reutericyclin

Reuterin is a low-molecular-mass broad spectrum antimicrobial substance originally described in *Lactobacillus reuteri*. It is active against several microorganisms including Gram-positive and Gram-negative bacteria, yeasts and fungi (TOBAJAS et al., 2007). Arqués et al. (2004) evaluated the antimicrobial spectrum of reuterin (8 AU/mL) and observed activity against *L. monocytogenes*, *S.*

aureus, *E. coli* O157:H7, *Salmonella enterica* subsp. *enterica* serotype Typhi, *Yersinia enterocolitica*, *Aeromonas hydrophila* and *Campylobacter jejuni*. According to Schaefer et al. (2010), reuterin acts by inhibiting bacterial growth by modification of thiol groups, probably in a large number of cell targets.

The production of reuterin is influenced by the presence of glycerol and low concentrations of glucose during the anaerobic growth under pH and redox conditions similar to the small and large intestine (CLEUSIX et al., 2008; SCHNÜRER; MAGNUSSON, 2005).

Reuterin is water-soluble, active in a wide range of pH, and it is resistant to proteolytic and lipolytic enzymes, which indicates its potential use as biopreservative in food (ÁVILA et al., 2014). Despite the addition of reuterin in food is not legislated, the addition of glycerol plus *L. reuteri* to produce reuterin *in situ* is an alternative, as demonstrated by Langa et al. (2013). Those authors detected the production of reuterin in dairy products added of glycerol and reuterin-producing *L. reuteri*.

Some strains of *L. reuteri* also produce reutericyclin, a highly hydrophobic antimicrobial compound active against Gram-positive bacteria (GÄNZLE, 2004; REIS et al., 2012).

1.3.4 Antifungal peptides

There is evidence of production of antifungal peptides by lactic acid bacteria (DALIÉ; DESCHAMPS; RICHARD-FORGET, 2010). Rouse et al. (2008) described four LAB species (*L. plantarum*, *Weissella confusa*, *P. pentosaceous* and *W. cibaria*) with antifungal activity due to the production of antimicrobial peptides. Those authors also showed that the *P. pentosaceous* was effective to prevent the growth of *Penicillium expansum* in apple models.

The occurrence of antifungal cyclic peptides produced by LAB has been reported in the literature. In 2002, Ström et al. identified antifungal compounds produced by *L. plantarum* MiLAB 393 as phenyllactic acid and cyclic peptides. Similarly, Magnusson et al. (2003) investigated the nature of the antifungal activity of several LAB and isolated active compounds produced by *Lactobacillus coryniformis* Si3 (phenyllactic acid and the cyclic peptides cyclo Phe-Pro and cyclo Phe-4-OH-Pro).

Together, these findings indicate the potential use of lactic acid bacteria and their metabolites in food preservation strategies.

1.4 Proteolytic system of lactic acid bacteria

LAB are fastidious saccharolytic bacteria that require numerous essential growth factors. In milk, there is a low concentration of peptides and free amino acids, and LAB growth rate depends on proteinases and peptidases to hydrolyze milk proteins to support their growth (SOUSA; ARDO; MCSWEENEY, 2001). The LAB proteolytic system comprises three main components: (i) cell wall proteinases that initiate the degradation of casein into oligopeptides (ii) peptide transporters that carry peptides into the cell, (iii) peptidases that hydrolyze intracellular peptides into smaller peptides or amino acids, which can be converted into various flavor compounds such as aldehydes, alcohols and esters (LIU et al., 2010; SAVIJOKI; INGMER; VARMANEN, 2006).

Milk provides all the essential amino acids for protein metabolism, and milk proteins are considered one of the highest quality proteins in human diet (KANWAR et al., 2009). Bovine milk is basically composed by caseins and whey proteins, which include immunoglobulins, α-lactalbumin, β-lactoglobulin, bovine serum albumin and lactoferrin (MILLS et al., 2011b). Native caseins, β-lactoglobulin and α-lactalbumin present in milk may have different biological activities such as ion carrier, retinol carrier, antioxidant, Ca^{2+} carrier, immunomodulatory and anticarcinogenic (KORHONEN; PIHLANTO, 2007). Moreover, these proteins may release bioactive peptides after hydrolysis by the action of LAB, with different activities such as antihypertensive, antioxidant, antimicrobial, immunomodulatory and mineral binding activities (BENKERROUM, 2010; DZIUBA; DZIUBA, 2014; PEPE et al., 2013; RICCI; ARTACHO; OLALLA, 2010).

On the other hand, consumption of milk protein (*e.g.*, α-lactalbumin and β-lactoglobulin) may represent a hazard for some individuals because of allergenicity. Cow milk allergy (CMA) especially affects children under 3 years of age and can reach 7% of incidence within this consumer group (CEBALLOS et al., 2009). In that context, fermented milk by LAB may help to reduce allergenicity. In 2010, Bu et al. demonstrated that fermentation of skim milk with *Lactobacillus helveticus* and *Streptococcus thermophilus* reduced allergenic potential of α-lactalbumin and β-

lactoglobulin. Similarly, in 2012, Ahmadova et al. demonstrated that proteolytic activity of *L. helveticus* A75 reduced the IgE binding ability of α_{S1}- and β-caseins. Thus, bacterial fermentation may be an alternative to reduce antigenic and/or allergenic properties of milk proteins due to the production of proteolytic enzymes by these organisms, specially the lactic acid bacteria (EL-GHAISH et al., 2011).

The association of the high nutritional value of milk with the effect of bacteria that positively modify milk proteins could result in new dairy products with benefits for human health and nutrition.

1.5 Lactic acid bacteria as probiotics

In early 20th century, Eli Metchnikoff observed the long life of Bulgarian and Eastern Europe peasants that had the habit of consuming fermented dairy products (AZIZPOUR et al., 2009). He isolated *Lactobacillus bulgaricus* (formerly *Bacillus bulgaricus*) from those fermented dairy products and postulated this microorganism was linked to health benefits (VIEIRA; TEIXEIRA; MARTINS, 2013). Eli Metchnikoff also hypothesized colonic bacteria produce toxic compounds involved in aging process. According to him, consumption of fermented milk would coat the colon with LAB, decreasing the intestinal pH and the number of 'putrefactive' bacteria in the gut (VERNA; LUCAK, 2010). Nowadays, the most commonly used probiotic strains belong to the *Bifidobacterium* and *Lactobacillus* genera (ROKKA; RANTAMAKI, 2010).

World Health Organization (WHO) and the Food and Agriculture Organization (FAO) define probiotic as "live microorganisms that when administered in adequate amounts confer health benefits to the host" (FOOD AND AGRICULTURE ORGANIZATION; WORLD HEALTH ORGANIZATION, 2002).

Probiotics may interfere with gut microbiota and re-establish it after antibiotic therapy, improve gut resistance to the colonization by pathogenic bacteria, produce antimicrobial substances into the intestinal lumen, increase of lactose digestion in lactose intolerant individuals, vitamins production, immune system stimulation and they may also alleviate symptoms of intestinal inflammatory diseases (SAAD et al., 2013; WALSH et al., 2014). Other potential probiotic effects may be related to decreased risk of intestinal disorders and reduction of serum cholesterol levels (PAVLOVIC; STANKOV; MIKOV, 2012).

Some authors have studied the role of probiotic in the modulation of immune system. Peng, Lin and Lin (2007) evaluated the effect of probiotic intake in rats for a period of 6 months, and showed that there was a decrease of the levels of specific anti-ovalbumin immunoglobulin E (IgE). These data indicates that probiotics may be beneficial in the treatment of food allergy, which is mainly mediated by IgE and leads to immediate type hypersensitivity or type I reaction (KUMAR et al., 2012). In addition, Pochard et al. (2002) evaluated the effect of probiotic on Th2 cytokine production in peripheral blood mononucleated cell (PBMC) of allergic patients. Those authors observed that probiotics reduced the production of interleukin-4 (IL-4) and interleukin-5 (IL-5) in PBMC only when the cells were stimulated with the specific allergen. Th2 cells have a key role in the immunity to extracellular parasites and all forms of allergic inflammatory responses, regulating B cell class switch to IgE by the production of IL-4 (PAUL; ZHU, 2010). Also, some probiotic strains may increase interleukin-12 (IL-12) production in dendritic cells and macrophages, converting a Th2 response into a Th1-dominated response in mice (KAWASHIMA et al., 2011). Thus, the Th1 cells produce interferon gama (IFN-γ) that suppresses antigen-specific IgE production in B cells (FUJIWARA et al., 2004). These data showed the potential of probiotic bacteria on modulating immune system, especially in allergic diseases. Otherwise, certain probiotic strains may increase the production of interleukin-10 (IL-10), an anti-inflammatory cytokine that downregulates the expression of Th1 pro-inflammatory cytokines (*e.g.* INF-γ), and decrease the production of the pro-inflammatory cytokines TNF and IL-8 (BAI et al., 2006; FERNANDEZ et al., 2011). INF-γ seems to play a key role in the pathogenesis of inflammatory bowel disease and other gastrointestinal disorders, thereby specific probiotics strains could be an additional strategy to alleviate symptoms in such conditions (CHIBA et al., 2006; REIFF; KELLY, 2010).

Probiotics may also contribute to protect against infectious agents, especially enteropathogens. Castillo et al. (2013) compared the ability of probiotic and non-probiotic lactobacilli strains to protect against *Salmonella enterica* serovar Typhimurium infection using a mouse model. Those authors reported that only the probiotic strain was able to protect against the pathogen by increasing intestinal barrier function and decreasing local inflammatory response. The intestinal barrier is a dense mucous layer containing secretory IgA cells, antimicrobial peptides and

dynamic junctional complexes that regulate the permeability between cells (OHLAND; MACNAUGHTON, 2010). Thus, probiotics may increase IgA secretion in these sites leading to an increased intestinal barrier function (PARVEZ et al., 2006).

An equilibrated gut flora also contributes to prevent the overgrowth of pathogens by the production of volatile fatty acids and decrease in pH of the luminal contents (SURAWICZ, 2003). However, antibiotic therapy can disrupt intestinal microbial imbalance and results in a variety of undesirable side effects, such as antibiotic-associated diarrhea (AAD), which occurs in as many as 30 % of patients (HEMPEL et al., 2012; KATZ, 2010). The mechanisms of AAD include disruption of the gut flora, effects of altered bacterial breakdown of carbohydrates and increased gastrointestinal effects of certain antibiotics (CREMONINI; VIDELOCK, 2013). In some patients, the pathogen *Clostridum difficile* grows to large numbers and produces toxins that cause colonic damage (SURAWICZ, 2003). Probiotics can positively contribute to prevent and alleviate symptoms of AAD, and treatment with *Lactobacillus rhamnosus* GG and *Saccharomyces boulardii* seemed to have the most significant efficacy (KATZ, 2010). There are several possible mechanisms by which probiotics may exert beneficial effects on AAD, including the synthesis of antimicrobial substances, competition for nutrients required for the growth of pathogens, competitive inhibition of adhesion of pathogens, and modification of toxins or toxin receptors (SONG et al. 2010).

It is important for strains used as probiotics to be able to adhere to epithelial cells, reduce adhesion of pathogenic bacteria on gut wall, tolerate gastrointestinal tract (GIT) conditions, production of antimicrobial substances, to be safe, noninvasive, noncarcinogenic and nonpathogenic, and to contribute on achieving a balanced intestinal microbiota (FOOD AND AGRICULTURE ORGANIZATION; WORLD HEALTH ORGANIZATION, 2002). In addition, the incorporation of probiotics in foods requires technological processes to maintain microbial viability under diverse conditions of acidity, oxygen level, presence of naturally or artificially added antimicrobial substances and nutrient availability (FORTIN et al., 2011; MINERVINI et al., 2012). One of the strategies to increase probiotic viability in food is the microencapsulation, where the bacterial cells are incorporated into a matrix to form microparticles. This approach not only protects the cells against harsh conditions in food, but also provides an additional resistance to adverse conditions in the GIT, leading to a site-specific release of probiotic cells in the intestine (BURGAIN et al.,

2011). It is also recommended to monitor probiotic functionality throughout all stages of production, storage and consumption of food products (VINDEROLA et al., 2011).

Overall, this literature review indicates there is an increasing demand for safe and high quality food, low in chemical preservatives and with health promoting properties. In this context, LAB are very attractive to improve food quality, safety and sensorial characteristics. LAB are part of the autochthonous microbiota of dairy products, and these represent good sources for the isolation of new strains of technological interest. Moreover, cheese and milk from different animals (*e.g.* cow, buffalo and goat) have a very diverse microbiota. The production of bacteriocins and other antimicrobial compounds by new isolates are of great interest, as these microorganisms (or their metabolites) may be applied in food biopreservation. There is also a current concern with regard to food allergenicity and cow milk allergy, which affects especially children under 3 years of age. Some LAB strains are able to hydrolyze milk proteins into small peptides and this may reduce allergenic potential of milk proteins, and also generate different bioactive peptides with health promoting properties. Moreover, data from literature indicate that LAB strains may present probiotic potential to benefit the health of consumers. Thus, it is important to search for new LAB isolates with potential to increase food safety and sensorial characteristics, and also to be used in the development of new products that can contribute to improve health and life quality.

2. Objectives

Objectives

The aim of this study was to isolated LAB strains from milk and cheese, with antimicrobial and proteolytic properties, for potential application in biopreservation and technological food application.

The specific goals of this study were as follows:

- Isolation of LAB from cow, buffalo and goat milk and cheese;
- Screening for antibacterial, antifungal and proteolytic activity among the isolates;
- Confirmation of the production of bacteriocin by the isolates that inhibited *Listeria monocytogenes*;
- Evaluation of the antibacterial spectrum of the isolated bacteriocinogenic strains;
- To partially purify the bacteriocin produced by strains with potential application in food products, to characterize the antimicrobial by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and to search for their genetic determinants using polymerase chain reaction (PCR);
- Confirmation of the antifungal activity in agar assays against *Penicillium expansum* and *Yarrowia lipolytica*, and also in a fermented milk model;
- Confirmation of the proteolytic activity on milk protein by electrophoresis analysis of fermented milk;
- To determine the conditions providing a better proteolytic activity among selected proteolytic LAB strains using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and high-performance liquid chromatography (HPLC);
- Evaluation of the biological activity of a fermented milk supernatant produced by proteolytic LAB strains on monocytes, to estimate the immunomodulatory effect;
- Evaluation of the probiotic potential of selected LAB with antimicrobial or proteolytic activities by testing the survival at low pH, digestive enzymes and bile salts, as well as the antibiotic resistance profile.

3. Screening for antimicrobial and proteolytic activities of lactic acid bacteria isolated from cow, buffalo and goat milk and cheese from southeast region of Brazil

Screening for antimicrobial and proteolytic activities of lactic acid bacteria isolated from cow, buffalo and goat milk and cheese from southeast region of Brazil

Abstract

From 156 milk and cheese samples of cow, buffalo and goat, 815 isolates were obtained on selective agars for lactic acid bacteria (LAB). The majority of them were Gram-positive cocci or bacilli, and negative for catalase production. Pure cultures of these new isolates were evaluated for antimicrobial activities by agar antagonism tests (spot-on-the-lawn assay), and for proteolytic activity on milk proteins by cultivation on agar plates (Brain Heart Infusion agar supplemented with skim milk). The most proteolytic isolates were also tested by cultivation in skim milk followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the fermented milk. Among the 815 tested isolates, four of them, identified by *16S rRNA* sequencing as *Lactobacillus paraplantarum* (strain FT259) and *Streptococcus uberis* (strains FT86, FT126, FT190) were bacteriocin producers, whereas four other strains identified as *Weissella confusa* FT424, *Weissella hellenica* FT476, *Leuconostoc citreum* FT671 and *Lactobacillus plantarum* FT723 showed high antifungal activity in preliminary assays. Complementary analyses showed that the most antifungal strain was *L. plantarum* FT723 that inhibited *Penicillium expansum* in modified MRS agar (De Man, Rogosa, Sharpe, without acetate) and fermented milk model. However, no inhibition was observed against *Yarrowia lipolytica*. Proteolytic activities were detected in 205 isolates by agar assay and 123 isolates with stronger proteolysis were submitted to confirmation of activity by SDS-PAGE. The proteolytic capacities of three most active isolates identified as *Enterococcus faecalis* (strains FT132 and FT522) and *Lactobacillus paracasei* FT700 were confirmed by SDS–PAGE, as visualized by the hydrolysis of caseins and whey proteins (β-lactoglobulin and α-lactalbumin). These results suggest potential applications of isolates in dairy food products.

Key-words: Lactic acid bacteria, milk, cheese, antimicrobial, proteolysis.

Isolamento de bactérias lácticas com atividade antimicrobiana e proteolíticas a partir de leite e queijo de vaca, búfala e cabra da região sudeste do Brasil

Resumo

A partir de 156 amostras de leite e queijo de vaca, búfalo e cabra, 815 isolados foram obtidos em ágares seletivos para bactérias lácticas. A maioria deles eram cocos ou bacilos Gram-positivos, e não produziam a enzima catalase. Culturas puras destes novos isolados foram avaliadas quanto a atividade antimicrobiana por testes de antagonismo em ágar (*spot-on-the-lawn assay*), e quanto a atividade proteolítica sobre as proteínas do leite por semeadura em placas de agar BHI (*Brain heart infusion*) adicionado de leite desnatado. Os isolados com maior atividade proteolítica foram avaliados também por cultivo em leite desnatado, seguido por eletroforese em gel de poliacrilamida com dodecil sulfato de sódio (*sodium dodecyl sulfate polyacrylamide gel electrophoresis*, SDS-PAGE) para a análise do leite fermentado. Entre os 815 isolados, quatro deles, identificados por sequenciamento do gene *16S rRNA* como *Lactobacillus paraplantarum* (linhagem FT259) e *Streptococcus uberis* (linhagens FT86, FT126 e FT190) foram caracterizados como produtores de bacteriocina, e quatro isolados identificados como *Weissella confusa* FT424, *Weisella hellenica* FT476, *Leuconostoc citreum* FT671 e *Lactobacillus plantarum* FT723 apresentaram elevada atividade antifúngica em ensaios preliminares. Análises complementares mostraram que o isolado com maior atividade antifúngica foi *L. plantarum* FT723, o qual inibiu o fungo indicador *Penicillium expansum* em ágar MRS modificado (De Man, Rogosa, Sharpe, sem acetato) e no modelo de leite fermentado. Entretanto, não houve inibição da multiplicação de *Yarrowia lipolytica*. Foram detectadas atividades proteolíticas em 205 isolados por cultivo em ágar, dos quais 123 isolados com maior atividade proteolítica foram submetidos a confirmação da atividade por SDS-PAGE. A atividade proteolítica de três isolados identificados como *Enterococcus faecalis* (linhagens FT1312 e FT522) e *Lactobacillus paracasei* FT700 foi confirmada por SDS-PAGE, como demonstrado pela hidrólise de caseínas e proteínas do lactosoro (β-lactoglobulina e α-lactalbumina). Estes resultados indicam o potencial de aplicação destes isolados em produtos lácteos.

Palavras-chave: bactérias láticas, leite, queijo, antimicrobianos, proteólise.

Criblage de bactéries lactiques isolées de laits de vache, de bufflonne et de chèvre et de différents fromages provenant du sud-est du Brésil pour la recherche d'activités antimicrobiennes et protéolytiques.

Résumé

Huit cent quinze isolats ont été obtenus sur des géloses sélectives pour les bactéries lactiques à partir de 156 échantillons de laits de vache, de bufflonne et de chèvre ainsi que de divers fromages. La majorité d'entre eux étaient des coques ou des bacilles à Gram positif, et ne produisaient pas de catalase. Des cultures pures de ces nouveaux isolats ont été évaluées pour leurs activités antimicrobiennes par des tests d'antagonisme sur gélose (*spot-on-the-lawn assay*), et pour leurs activités protéolytiques sur les protéines du lait par culture sur gélose (milieu *Brain Heart Infusion* agar supplémenté de lait écrémé). Les isolats les plus protéolytiques ont été également évalués par culture dans du lait écrémé suivie d'une électrophorèse sur gel de polyacrylamide en condition dénaturante par l'ajout de dodécyl sulfate de sodium (*sodium dodecyl sulfate polyacrylamide gel electrophoresis*, SDS-PAGE). Parmi les 815 isolats testés, quatre d'entre eux, identifiés par séquençage du *16S rRNA* comme *Lactobacillus paraplantarum* (souche FT259) et *Streptococcus uberis* (souches FT86, FT126, FT190) se sont révélés être producteurs de bactériocines, alors que quatre autres isolats identifiés comme *Weissella confusa* FT424, *Weissella hellenica* FT476, *Leuconostoc citreum* FT671 et *Lactobacillus plantarum* FT723 ont montré une forte activité antifongique lors d'essais préliminaires. Des analyses complémentaires ont montré que la souche la plus antifongique était *L. plantarum* FT723, laquelle a inhibé *Penicillium expansum* sur gélose MRS modifiée (De Man, Rogosa, Sharpe sans acétate) et dans un modèle de lait fermenté. Cependant, aucune inhibition n'a été observée contre *Yarrowia lipolytica*. Des activités protéolytiques ont été détectées dans 205 isolats par culture sur géloses supplémentées en lait écrémé. Parmi ces isolats, les 123 les plus protéolytiques ont vu leur activité protéolytique confirmée par SDS-PAGE. Les activités des trois isolats les plus protéolytiques *Enterococcus faecalis* (souches FT132 et FT522) et *Lactobacillus paracasei* FT700, ont été confirmées par la visualisation de la digestion des caséines et des protéines du lactosérum (β-lactoglobuline et α-lactalbumine) sur SDS-PAGE. Ces résultats suggèrent des applications potentielles de ces isolats dans les produits alimentaires tels que les produits laitiers fermentés.

Mots-clés: bactéries lactiques, lait, fromage, antimicrobiens, activités protéolytiques.

1. Introduction

Lactic acid bacteria (LAB) can be isolated from different sources such as milk, meat, decomposing plant material and mammalian gastrointestinal tract, and are industrially important in different food-fermentation processes (TEUSINK; SMID, 2006). The lipolytic, proteolytic and glycolytic enzymes of LAB are important in cheese preservation and in flavor production (NAVIDGHASEMIZAD et al., 2009). Moreover, LAB produce several antimicrobial compounds such as organic acids, diacetyl, acetoin, hydrogen peroxide, reuterin, antifungal peptides and bacteriocins, which make these bacteria interesting for food biopreservation (GÁLVEZ et al., 2007). Thus, the aim of this study was to isolate and screen LAB from cow, buffalo and goat milk and cheeses for potential application in biopreservation and technological improvement of dairy products. The Gram-positive and catalase negative isolates were initially screened for production of antibacterial and antifungal compounds, and for the presence of proteolytic activity on milk proteins. Next, the most active isolates were submitted to specific tests to confirm the production of bacteriocins, the antifungal activity in fermented milk, and hydrolysis of milk proteins. The selected isolates were identified by *16S rRNA* gene sequencing and used in studies described in the next chapters.

2. Materials and methods

2.1 Strains and culture conditions

Bacteria and fungi from different culture collections were used as reference strains, and their names and culture conditions are listed in Table 3.1. When needed, the anaerobic generation system AnaeroGen^{TM} (Oxoid, UK) was used.

Table 3.1 Strains used in this study as biological indicators

Organism	Strain	Culture Media	Incubation temperature
Bacteria			
Carnobacterium maltaromaticum A9b-	DIFR[a]	BHI[b]	25 °C
Cronobacter sakazakii	ATCC[c] 29004	BHI	37 °C
Enterobacter aerogenes	CDC[d] 1535	BHI	37 °C
Escherichia coli	CDC 02A.2B	BHI	37 °C
Klebsiella pneumoniae	ATCC 10031	BHI	37 °C
Lactobacillus sakei	ATCC 15521	MRS[e]	25 °C
Listeria innocua	ATCC 33090	BHI	37 °C
Listeria monocytogenes	IAL[f] 633	BHI	37 °C
Listeria monocytogenes	ATCC 19115	BHI	37 °C
Proteus mirabilis	CDC 305	BHI	37 °C
Pseudomonas aeruginosa	ATCC 14502	BHI	37 °C
Salmonella enterica subsp. *enterica* serovar Enteritidis	ATCC 13076	BHI	37 °C
Staphylococcus aureus	ATCC 29213	BHI	37 °C
Staphylococcus epidermidis	ATCC 14990	BHI	37 °C
Streptococcus thermophilus	STANDA[g]	M17	42 °C
Fungi			
Kluyveromyces lactis	ATCC 56498	YEM[h]	25°C
Penicillium expansum	UBOCC1.08.102[i]	PDA[j]	25 °C
Yarrowia lipolytica	UBOCC 2.11.004	YEM	25 °C

[a] Nilsson et al. (2004). Danish Institute for Fisheries Research, Department of Seafood Research, Lyngby, Denmark.
[b] Brain Heart Infusion.
[c] American Type Culture Collection, Manassas, USA.
[d] Centers for Disease Control and Prevention, Atlanta, USA.
[e] De Man, Rogosa, Sharpe.
[f] Instituto Adolfo Lutz, São Paulo, Brazil.
[g] Commercial starter STANDA laboratories, Caen, France.
[h] Yeast extract and malt.
[i] Université de Brest Culture Collection and Laboratory for Microbiology of the Faculty of Sciences of Ghent University.
[j] Potato Dextrose Agar.

2.2 Samples

One hundred twenty-seven samples of cow, buffalo and goat milk were collected in the region of Ribeirão Preto (São Paulo, Brazil) in sterilized packages (Whirl-Pak, Nasco, USA), taken to the laboratory in insulated box and stored under refrigeration for analysis within a maximum of 24 h after sampling. Each milk sample was collect from a single animal, and each type of milk (cow, buffalo and goat) was collected in a single farm. Twenty-nine samples of cow, buffalo and goat cheeses commercially available (Minas, Mozzarella, Emmental, Gruyère, Gouda, Brie, Parmesan, Provolone, Gorgonzola, Reino, Curd and Ricotta) were acquired in the city of Ribeirão Preto (São Paulo, Brazil) and stored under refrigeration for up to 48 h before analysis.

2.3 Isolation and preliminary identification of lactic acid bacteria

Milk (10 mL) and cheese (25 g) samples for LAB isolation were ten-fold diluted with 0.1 % w/v bacteriological peptone solution (Oxoid) and homogenized for 1 min in a laboratory blender (BagMixer, Interscience, France). Serial dilutions were plated on De Man, Rogosa, Sharpe agar (MRS, Oxoid, pH 6.2) for the isolation of LAB, and on LAMVAB agar (MRS, Oxoid, plus vancomycin 20 mg/L, Sigma-Aldrich, USA, adjusted to pH 5.0) for the isolation of lactobacilli (HARTEMINK; VAN LAERE; ROMBOUTS, 1997). Inoculated plates were incubated at 25 °C for 72 h under anaerobic atmosphere. After incubation, colonies of different morphologies were selected and reisolated at least three times on MRS agar. Pure isolates were inoculated in BHI broth (Brain Heart Infusion, Oxoid), and incubated for 24 h at 25 °C. The new isolates were stored in BHI broth with 20 % v/v glycerol at -80 °C. Preliminary identification was done by Gram staining and testing for the absence of catalase. For further tests, the rod-shaped isolates were propagated in MRS broth and coccus-shaped isolates were propagated in BHI broth (Oxoid).

2.4 Evaluation of antibacterial activity

The preliminary evaluation of the antibacterial activity of Gram-positive and catalase negative new isolates was performed according Lewus, Kaiser and Montville

(1991). Two microliters of a 24 h LAB culture in MRS or BHI broths of each isolate were applied as spots on a TSA-ye agar plate (Trypticase soy agar supplemented with 0.6 % w/v of yeast extract, Oxoid) and incubated at 25 °C for 48 h under anaerobic atmosphere. TSA-ye medium was used instead of MRS in order to reduce production of organic acids that could interfere with bacteriocin detection. After incubation, the plates were overlaid with soft BHI agar (BHI broth with 0.8 % w/v bacteriological agar) inoculated at 10^6 CFU/mL of *Listeria monocytogenes* IAL 633 (indicator strain), followed by incubation at 37 °C for 24 h. The production of antibacterial compounds was detected by zones of growth inhibition around the inoculation spot. Isolates showing the most effective antilisterial activity were also tested against Gram-positive and Gram-negative indicator bacteria (Table 3.1) to determine their inhibitory spectrum.

The proteinaceous nature of the antibacterial substances produced by the most effective strains was evaluated according to Lewus, Kaiser and Montville (1991) with slight modifications. Two microliters of each 24 h antibacterial strain culture in MRS broth (for rod-shaped isolates) or BHI broth (for coccus-shaped) isolates were applied as spots on a TSA-ye agar plate (Trypticase soy agar supplemented with 0.6% w/v of yeast extract) and incubated at 25 °C for 48 h under anaerobic atmosphere. Two millimeter wells were made in the agar plates next to the producers' spot, and filled with 20 µL of 20 mg/mL aqueous solutions of the protease type XIV from *Streptomyces griseus* (Sigma-Aldrich), proteinase K from *Tritirachium album* (Sigma-Aldrich) or α-chymotrypsin from bovine pancreas (Sigma-Aldrich). Sterilized water was used as a negative control. The plates were incubated at 30 °C for 2 h and each plate was overlaid with soft BHI agar (BHI with 0.8 % w/v agar) inoculated with 10^6 CFU/mL of *L. monocytogenes* IAL 633, followed by incubation at 37 °C for 24 h. The absence of inhibitory halo in the presence of proteolytic enzymes was indicative of the production of antibacterial peptides (bacteriocins).

2.5 Preliminary evaluation of antifungal activity

The first evaluation of the antifungal activity of Gram-positive and catalase negative isolates was performed according Delavenne et al. (2012). For each isolate, two microliters of a 24 h culture in MRS or BHI broths were applied as spots on MRS agar and incubated at 25 °C for 48 h under anaerobic atmosphere. The plates were

overlaid with soft YEM agar (Yeast extract and malt agar, Difco, USA, supplemented with 0.8% w/v agar) inoculated at 10^6 CFU/mL of *Kluyveromyces lactis* ATCC 56498 and incubated at 25 °C for 48 h. The production of antifungal compounds was detected by inhibition growth zones of *K. lactis*. Isolates showing larger inhibition zones of the indicator were selected for further investigation.

2.5.1 Confirmation of antifungal activity in modified MRS agar

Confirmation of the antifungal activity of pre-selected antifungal isolates was done on modified MRS agar with low sugar concentration and without acetate, as organic acids increase antifungal activity (DELAVENNE et al., 2012; SCHILLINGER; VILLARREAL, 2010). The modified MRS was composed as follows: peptone 10 g/L, meat extract 10 g/L, yeast extract 5 g/L, glucose 1.25 g/L, fructose 1.25 g/L, sucrose 1.25 g/L, maltose 1.25 g/L, glycerol 150 mM, Tween 80 1.08 g/L, dipotassium phosphate 2 g/L, ammonium citrate 2 g/L, magnesium sulfate 0.2 g/L, manganese sulfate 0.05 g/L, bromocresol green 20 mg/L, agar 7 g/L. Overnight cultures of LAB in MRS broth were inoculated at *ca*. 10^7 CFU/mL in modified MRS broth and incubated at 25 °C or 37 °C for 24 h, depending on the strain tested. Strains were inoculated once more at 10^7 CFU/mL in modified MRS and incubated at optimal temperature for 18 h or until reach approximately 1.0 of optical density (OD_{600nm}). Ten microliters of each culture were deposited in 24-wells plates, and mixed with 1 mL of modified soft MRS agar (modified MRS broth supplemented with 0.8 % w/v agar). After incubation at optimal temperature for 24 h, 100 spores per well or 100 CFU per well of the indicators, respectively *Penicillium expansum* UBOCC 1.08.102 or *Yarrowia lipolytica* UBOCC 2.11.004, were applied and incubated at 25 °C for 2 weeks.

2.5.2 Evaluation of antifungal activity in fermented milk

Skim milk (Délisse UHT, France) supplemented with 4 % w/v milk powder and 0.06 % w/v litmus (RAL Reactifs, France) was heated at 85 °C for 30 min, cooled on ice until 45 °C, and distributed in 24-well plates (2 mL in each well). Strains suspended in MRS broth were washed with 0.85 % NaCl solution and inoculated in milk at 10^7 CFU/mL at the same time as *Streptococcus thermophilus* (10^7 CFU/mL) that was used as starter. The inoculated milk was then incubated at 42 °C and after

24 h of incubation the excess of serum was withdrawn from the curd with a pipette. Next, the two indicator strains (100 spores per well of *P. expansum* and 100 CFU per well of *Y. lipolytica*) were inoculated over the curd surface and the fermented milk was reincubated at 10 °C for 2 weeks.

2.6 Evaluation of proteolytic activity

The evaluation of proteolytic activities of Gram-positive and catalase negative isolates was initially performed on BHI agar supplemented with skim milk. Isolates showing proteolytic activity were further cultivated in skim milk, followed by in polyacrylamide gel electrophoresis analysis, as described below.

2.6.1 Proteolytic activity on agar

Proteolytic activity of Gram-positive and catalase negative isolates was first evaluated in BHI agar supplemented with 33 % v/v skim milk powder (Oxoid) as described by Pailin et al. (2001). For this, 5.55 g of BHI and 2.25 g of bacteriological agar were dissolved in 100 mL of purified water, and 5 g of skim milk powder were dissolved in 50 mL of purified water. These flasks were autoclaved separately at 121 °C for 15 min. Both solutions were cooled to 50 °C, mixed in a single flask, and distributed in Petri dishes. Isolates were inoculated as 2 µL spots on the plates and incubated at 25 °C for 48 h under anaerobic atmosphere. The occurrence of translucent halos around the colonies was indicative of proteolytic activity.

2.6.2 Proteolytic activity on skim milk

The proteolytic activity of isolates that presented intense activity on agar plates was also evaluated in skim milk according to El-Ghaish et al. (2010). One hundred microliters of an overnight LAB culture in BHI or MRS broths were centrifuged at 10,000 x *g* for 10 minutes, and the cells were washed with phosphate buffered saline (PBS, 8.0 g/L NaCl, 0. 2 g/L KCl, 1.44 g/L Na_2HPO_4 and 0.24 g/L KH_2PO_4, pH 7.2, all components were purchased from Synth, Brazil). The cell pellet was resuspended in 1 mL of sterilized skim milk (Oxoid) and incubated for 24 h at 25 °C or 37 °C according to the optimal incubation temperature for each isolate. After the incubation,

10 µL of the fermented milk (concentration of *ca*. 10^8 CFU/mL) were diluted in 90 µL of buffer containing 4 % w/v SDS (sodium dodecyl sulfate, Synth), 3 % v/v mercaptoethanol (Sigma-Aldrich), 10 % v/v glycerol (Synth), 50 mM tris-HCl pH 6.8 (Serva, Germany) and 0.07 % w/v bromophenol blue (Bio-Agency, Brazil). The samples were heated at 100 °C for 3 minutes and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as described below.

Samples were analyzed in a vertical electrophoresis system (Mini-PROTEAN II Electrophoresis Cell, Bio-rad Laboratories, USA) using a polyacrylamide gel (12 % resolving gel and 3.2 % stacking gel), as described by El-Ghaish et al. (2010). Solutions and reagents used to prepare the gels are presented in Table 3.2. The running buffer was prepared with 50 mM tris base (Serva), 0.384 M glycine (Sigma-Aldrich) and 0.1 % w/v SDS. The electrophoretic separation was carried out with 10 mA for the stacking gel and 20 mA for the resolving gel, followed by staining with 0.2 % w/v Coomassie Brilliant Blue R-250 (Sigma-Aldrich), prepared with 30 % v/v ethanol (Synth) and 5 % v/v glacial acetic acid (Synth). The gels were destained with an aqueous solution containing 30 % v/v ethanol and 5 % v/v glacial acetic acid, and photographed (MiniBis UV, DNA Bio-Imagings Systems, Israel).

Table 3.2 Composition of polyacrylamide gels used for SDS-PAGE analyses of fermented milk produced LAB. Values are presented for plates with 0.75 mm spacers

Component	Stacking gel (3.2 %)	Resolving gel (12 %)
Acrylamide-bis[(1)] (29:1) 40 %	200 µL	2400 µL
Tris-HCl[(2)] 2 M, pH 8.8	0	1330 µL
Tris-HCl[(2)] 0.5 M, pH 6.8	300 µL	0
Water	2000 µL	4270 µL
SDS[(3)] 10 %	25 µL	80 µL
TEMED[(4)]	4 µL	16 µL
Ammonium persulfate[(2)] 10 % (aqueous solution)	20 µL	80 µL
Total	**2549 µL**	**8176 µL**

[(1)] Supplier: Sigma-Aldrich, USA.
[(2)] Supplier: Synth, Brazil.
[(3)] Sodium dodecyl sulfate (Synth).
[(4)] N,N,N′,N′-Tetramethylethane-1,2-diamine (Sigma-Aldrich).

2.7 Genotypic identification

Genotypic identification of 33 bacterial isolates (Table 3.5) with antimicrobial or proteolytic activity was performed by *16S rRNA* gene sequencing. Genomic DNA was extracted and purified with an Illustra Bacteria Genomic Prep Mini Spin Kit (GE Life Sciences, Sweden) and used as template for genotypic identification. Polymerase chain reaction (PCR) was performed with 84 µL of Taq Platinum Blue (Invitrogen, USA), 30 pmol of primer 27F (Invitrogen), 30 pmol of primer 1492R (Invitrogen) and 300 ng of genomic DNA, in a total volume of 100 µL (LANE, 2001; TURNER et al., 1999). PCR cycles were performed with an initial denaturation step at 94 °C for 1 minute, followed by annealing at 55 °C for 1 minute, and extension at 72 °C for 2 minutes, for a total of 30 cycles. PCR products were purified using Illustra GFX PCR DNA and Gel Band Purification (GE Life Sciences). The purified PCR products were sequenced using ABI 3730 DNA Analyzer (Applied Biosystems, USA) with BigDye Terminator v3.1 Cycle Sequencing Reagent (Applied Biosystems) at The Human Genome Research Center (HGRC) of the University of São Paulo, São Paulo, Brazil. Sequences were analyzed using Chromas Lite 2.1 (Technelysium, South Brisbane, Australia) and compared with sequences available in GenBank, with the National Center for Biotechnology Information BLASTN search program (http://www.ncbi.nlm.nih.gov/BLAST). Isolates belonging to the *Lactobacillus plantarum* group (*L. plantarum*, *Lactobacillus paraplantarum* and *Lactobacillus pentosus*) were also identified with species-specific primers planF, pentF, paraF and pREV (TORRIANI; FELIS; DELLAGLIO, 2001) targeting the *rec*A gene. The thermal cycle used was denaturation at 94 °C for 30 s, annealing at 56 °C for 10 s, and extension at 72 °C for 30 s for a total of 30 cycles. PCR products of 107, 218 and 318 bp corresponded respectively to *L. paraplantarum*, *L. pentosus* and *L. plantarum*. The oligonucleotide sequences of the primers used in this study are shown in Table 3.3. LAB isolates that could not be distinguished by *16S rRNA* gene sequencing were submitted to PCR with species-specific primers, as described in Table 3.3. The thermal cycle was used as described in each reference, with slight modifications on annealing temperature as shown. PCR products were analyzed by electrophoresis at 100 V for 60 minutes using 1 % w/v agarose gel in TAE buffer. Next, the PCR products in the gel were stained with 0.5 µg/mL ethidium bromide solution for 20

minutes, and the bands were visualized under ultraviolet (UV) light (MiniBis UV, DNA Bio-Imagings Systems, Israel).

Table 3.3 Oligonucleotide sequences of the primers used for identification of LAB isolates by *16S rRNA* gene sequencing and PCR with species-specific primers

Primer	Oligonucleotide sequence (5'-3')	Target species	Annealing temperature (°C)	Reference
27F	AGA GTT TGA TCM TGG CTC AG	Universal (*16S rRNA*)	55	LANE, 2001
1492R	GCT TAC CTT GTT ACG ACT T	Universal (*16S rRNA*)		TURNER et al., 1999
planF	CCG TTT ATG CGG AAC ACC TA	*L. plantarum*	56	TORRIANI; FELIS; DELLAGLIO, 2001
pentF	CAG TGG CGC GGT TGA TAT C	*L. pentosus*		
paraF	GTC ACA GGC ATT ACG AAA AC	*L. paraplantarum*		
pREV	TCG GGA TTA CCA AAC ATC AC	*L. plantarum* group		
EfaecalF	ATC AAG TAC AGT TAG TCT	*E. faecalis*	55	DUTKA-MALEN; EVENS; COUVARLIN, 1995
EfaecalR	ACG ATT CAA AGC TAA CTG	*E. faecalis*		
EfaeciumF	TAG AGA CAT TGA ATA TGC C	*E. faecium*		
EfaeciumR	TCG AAT GTG CTA CAA TC	*E. faecium*		
hi1	CTT TCT GAT ATG GAT GCT GTC	*E. hirae*	56	JACKSON; FEDORKA-CRAY; BARRETT, 2004
hi2	TAA ATT CTT CCT TAA ATG TTG	*E. hirae*		

casei	TGC ACT GAG ATT CGA CTT AA	*L. casei*	50	WARD; TIMMINS, 1999
para	CAC CGA GAT TCA ACA TGG	*L. paracasei*		
rham	TGC ATC TTG ATT TAA TTT TG	*L. rhamnosus*		
Y2	CCC ACT GCT GCC TCC CGT AGG AGT	*L. casei* group		
LcurR	TTG GTA CTA TTT AAT TCT TAG	*L. curvatus*	53	BERTHIER; EHRLICH, 1998
LsakeR	ATG AAA CTA TTA AAT TGG TAC	*L. sakei*		
16FOR	GCT GGA TCA CCT CCT TTC	*L. sakei* group		
LmesF	AAC TTA GTG TCG CAT GAC	*Lc. mesenteroides*	56	LEE; PARK; KIM, 2000
LmesR	AGT CGA GTT ACA GAC TAC AA	*Lc. mesenteroides*		

E.: *Enterococcus*; *L.*: *Lactobacillus*; *Lc* : *Leuconostoc.*

3. Results and discussion

In this research, for selecting LAB of technological interest with proteolytic and/or antimicrobial activities with potential applications in the production of new dairy products, isolates were obtained from samples of cow, buffalo and goat milk and cheese.

3.1 Characterization of isolates

From the 127 samples collected of cow (n = 80), buffalo (n = 35) and goat (n = 12) milk, and the 29 samples of cow (n = 17), buffalo (n = 5) and goat (n = 7) cheeses, a total of 815 isolates were obtained from MRS and LAMVAB agars. Among the collected raw milk samples, 52 samples from cow milk (65 %), five from buffalo milk (14 %) and 12 from goat milk (100 %) did not yield colonies on MRS

neither LAMVAB agars. Cow milk samples showed positive bacterial growth in MRS only, whereas bacterial growth for buffalo milk samples was observed on both MRS and LAMVAB agars. In Figure 3.1 are represented the mean populations and standard deviations obtained among the milk samples that were positive for LAB isolates (samples with bacterial population below the quantification threshold of 2.4 log CFU/mL were not considered).

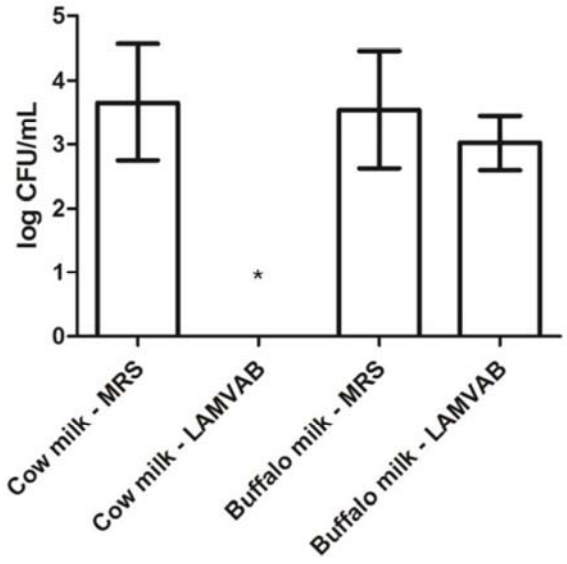


Figure 3.1 Lactic acid bacteria and lactobacilli population enumerated, respectively, on MRS and LAMVAB agars, for milk samples. Bars represent the mean ± standard deviation. The samples that were below quantification threshold (2.4 log CFU/mL) were not considered in the calculation of the mean and standard deviation. * No CFU was detected.

Average LAB population enumerated on MRS agar was 3.6 ± 0.9 log CFU/mL in cow milk and 3.5 ± 0.9 log CFU/mL in buffalo milk. Similarly, an average of 3.0 ± 0.4 log CFU/mL as found in buffalo milk samples using LAMVAB agar plates. Delavenne et al. (2012) evaluated the presence of LAB in cow, goat and sheep milk samples. They reported an average bacterial population of 3.8 and 4.4 log CFU/mL, respectively for cow and goat milk samples, using MRS agar acidified to pH 5.5. In the present work, cow milk samples analyzed in MRS agar (pH 6.2) presented a similar average bacterial population of 3.6 log CFU/mL, however, no LAB were isolated from goat milk samples. Delavenne et al. (2012) also used the LAMVAB agar to isolate LAB, and detected an average bacterial population of 3.9 log CFU/mL in both cow and goat milk samples. Microbial composition of goat milk may vary along

seasons and depending on lactation period. They also can be affected by other factors as the type of feeding, hygienic conditions of milking, breeders' practices and location of the farm (CALLON et al., 2007; FOSCHINO ET AL., 2002). This may explain the difference between bacterial population in cow and goat milk samples described in the present study.

Total bacterial population detected on MRS agar (LAB) and LAMVAB agar (lactobacilli) for the analysis of cheese samples are presented in Table 3.4. Only one sample (Provolone cheese, sample no. 72) presented bacterial population below detection limit of the method (2.4 log CFU/mL) for both media.

Table 3.4 Cheese samples analyzed during the screening for lactic acid bacteria isolates and bacterial populations enumerated on MRS (LAB) and LAMVAB (lactobacilli) agars. Detection limit = 250 CFU/g or 2.4 log CFU/g

Sample number	Description	Culture media (log CFU/g)	
		MRS	LAMVAB
2	Cow cheese (“Minas”)	8.6	7.9
3	Cow cheese (Mozzarella)	6.2	6.0
65	Cow cheese (Mozzarella)	7.9	8.0
66	Cow cheese (“Minas”)	8.3	7.9
67	Cow cheese (Emmental)	6.2	<2.4
68	Cow cheese (Gruyère)	7.3	6.5
69	Cow cheese (Gouda)	7.5	6.8
70	Cow cheese (Brie)	7.6	6.0
71	Cow cheese (Parmesan)	6.5	6.3
72	Cow cheese (Provolone)	<2.4	<2.4
73	Cow cheese (Gorgonzola)	7.1	6.6
74	Cow cheese (Reino)	6.3	4.6
91	Cow cheese (Curd cheese)	5.1	5.3
92	Cow cheese (Provolone)	7.9	7.7
93	Cow cheese (Parmesan)	6.4	5.3
94	Cow cheese (“Minas”)	8.6	8.2
95	Cow cheese (“Minas”)	8.3	8.3
133	Buffalo cheese	8.6	8.0
134	Buffalo cheese (Mozzarella)	5.4	4.8
135	Buffalo cheese (Mozzarella)	6.8	6.8
136	Buffalo cheese (Ricotta)	6.0	6.3
137	Buffalo cheese (Mozzarella)	5.3	5.3
150	Goat cheese	4.9	3.6
151	Goat cheese (with spices)	7.8	8.2
152	Goat cheese	8.6	8.5
153	Goat cheese (ripened)	5.1	4.1
154	Goat cheese	7.8	7.3
155	Goat cheese	8.1	6.6
156	Goat cheese	6.2	6.1

Gram-positive cocci and rods were isolated from 28 out of 29 cheese samples (96.5 %) in MRS and LAMVAB agars. The mean of bacterial population enumerated in MRS agar for cow, buffalo and goat cheeses were, respectively, 7.2 ± 1.0 log CFU/g, 6.4 ± 1.4 log CFU/g and 6.9 ± 1.5 log CFU/g. Similarly, the mean of bacterial population enumerated on LAMVAB agar for cow, buffalo and goat cheeses were, respectively, 6.8 ± 1.2 log CFU/g, 6.2 ± 1.3 log CFU/g and 6.3 ± 1.9 log CFU/g. Asteri et al. (2009) analyzed samples of the Greek cheese Formaella in MRS agar, and obtained bacterial populations between 6.4 and 7.0 log CFU/mL. Similarly, in the present study, it was obtained an average bacterial population of 7.0 log CFU/g for cheese samples, enumerated on MRS agar. In Figure 3.2 are represented the mean populations and standard deviations obtained for the cheese samples positive for LAB isolates (samples with bacterial population below the quantification threshold of 250 CFU/g or 2.4 log CFU/g were not considered).

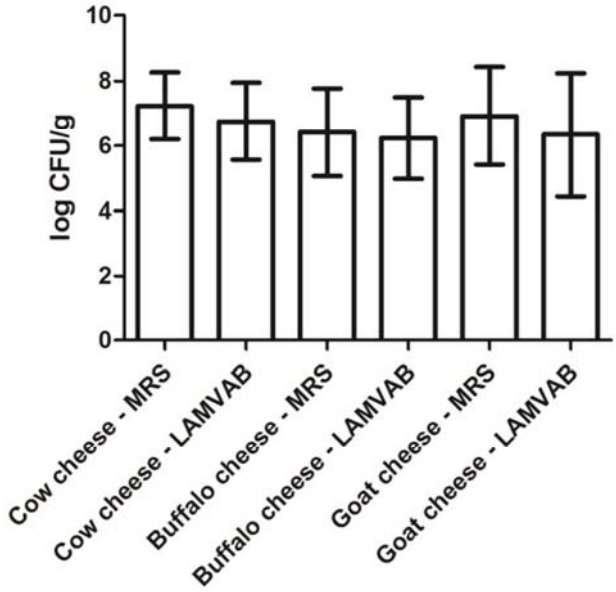


Figure 3.2 Lactic acid bacteria and lactobacilli population enumerated, respectively, on MRS and LAMVAB agars, for cheese samples. Bars represent the mean ± standard deviation. The samples that were below quantification threshold (250 CFU/g) were not considered in the calculation of the mean and standard deviation.

In the present work, LAMVAB agar was used for selective isolation of lactobacilli, based on low pH (5.0) and intrinsic resistance of lactobacilli to vancomycin (HARTEMINK et al., 1997). Most isolates obtained in LAMVAB agar were rod-shaped, but some cocci-shaped bacteria also grew in this medium. The

cocci were further identified as belonging to the genus *Weissella* (Table 3.5). According to Harwood et al. (2001), species belonging to the genera *Leuconostoc*, *Pediococcus* and *Weissella* are also intrinsically resistant to vancomycin, which can explain the isolation of *Weissella* spp. on LAMVAB agar.

A total of 815 colonies were purified from MRS and LAMVAB agar plates, and most of them were Gram-positive and catalase negative isolates, considered a presumptive identification for LAB (AXELSSON, 1993). They were selected for further evaluation of antimicrobial and proteolytic activities, and identified by *16S rRNA* gene sequencing using primers 27F and 1492R. The identification of some strains was also confirmed by species-specific primers (Table 3.3).

Table 3.5 Identification of bacteria isolated from milk and cheese, selected based on antimicrobial and/or proteolytic activity

Strain	Identification[1]	Characteristic[2]	GenBank[3] accession no.
Cow milk			
FT86	*Streptococcus uberis*	Antibacterial	KM207805
FT106	*Enterococcus faecium*	Antibacterial	N/A[4]
FT126	*Streptococcus uberis*	Antibacterial	KM207806
FT132	*Enterococcus faecalis*	Proteolytic	KM207832
FT190	*Streptococcus uberis*	Antibacterial	KM207807
Cow cheese			
FT13	*Lactococcus lactis*	Proteolytic	KM207828
FT23	*Lactobacillus plantarum*	Antifungal / Proteolytic	KM207829
FT58	*Lactobacillus fermentum*	Antifungal / Proteolytic	KM207831
FT135	*Lactobacillus paracasei*	Proteolytic	KM207833
FT144	*Lactobacillus paracasei*	Proteolytic	KM207834
FT179	*Lactobacillus paracasei*	Antibacterial	KM207835
FT218	*Lactobacillus rhamnosus*	Antibacterial	KM207836
FT232	*Lactobacillus paracasei*	Proteolytic	KM207837
FT255	*Lactococcus garvieae*	Proteolytic	KM207838
FT259	*Lactobacillus paraplantarum*	Antibacterial	KF569800
FT286	*Lactococcus lactis*	Proteolytic	KM207840

Buffalo milk			
FT351	*Weissella paramesenteroides*	Proteolytic	KM207841
FT359	*Lactococcus lactis*	Proteolytic	KM207813
FT369	*Weissella paramesenteroides*	Antifungal	KM207813
FT417	*Lactococcus lactis*	Proteolytic	KM207814
FT424	*Weissella confusa*	Antifungal	KM207815
FT442	*Weissella paramesenteroides*	Antifungal	KM207816
FT476	*Weissella hellenica*	Antifungal / Proteolytic	KM207809
FT522	*Enterococcus faecalis*	Proteolytic	KM207818
FT541	*Weissella confusa*	Antifungal	KM207819
Buffalo cheese			
FT609	*Lactococcus lactis*	Antibacterial	KM207821
FT655	*Enterococcus hirae*	Antibacterial / Proteolytic	KM207822
FT664	*Leuconostoc mesenteroides*	Antifungal	KM207823
FT671	*Leuconostoc citreum*	Antifungal / Proteolytic	KM207810
Goat cheese			
FT697	*Lactococcus lactis*	Proteolytic	KM207824
FT700	*Lactobacillus paracasei*	Proteolytic	KM207811
FT723	*Lactobacillus plantarum*	Antifungal	KM207812
FT771	*Lactobacillus plantarum*	Antifungal	KM207826

(1) Identification by *16S rRNA* gene sequencing. Additional identification by polymerase chain reaction (PCR) with species-specific primers (Table 3.3) was performed when necessary.
(2) Preliminary evaluation of antibacterial, antifungal and proteolytic activities as described, respectively, in items 2.4, 2.5 and 2.6.1.
(3) *16S rRNA* sequences available at http://www.ncbi.nlm.nih.gov/genbank/.
(4) N/A: not applicable - the species was determined by PCR using species-specific primers.

Most of the isolates were identified as belonging to the genus *Lactobacillus*. Other isolates were also identified as belonging to the genera *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Streptococcus* and *Weissella* (Figure 3.3).

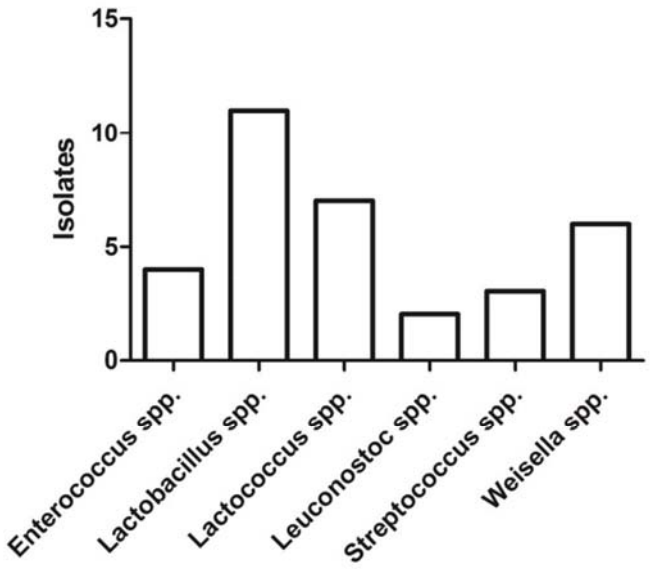


Figure 3.3 Lactic acid bacteria genera isolated from milk and cheese samples.

In 2012, Yang et al. isolated 137 LAB from cheese and yogurts and identified 28 strains that presented antimicrobial activity, using MRS and M17 agars. Those authors observed a high occurrence of the genus *Enterococcus* (85.7 %, but all of them were *E. faecium*), followed by 7.1 % of *Streptococcus* and 7.1 % of *Lactobacillus*. In the present work, the most frequently isolated genus was *Lactobacillus*. This different result may be due to the different composition of samples, and also by the use of a specific medium for the isolation of lactobacilli (LAMVAB).

3.1.1 Evaluation of antibacterial activity

The ability to inhibit the growth of *L. monocytogenes* IAL 633 (indicator strain) was observed for 9 isolates by the spot-on-the-lawn assay. However, only 4 isolates were selected for further assays because they showed higher inhibitory activity against *L. monocytogenes* IAL 633, as demonstrated by the diameter of inhibition zone larger than 10 mm. The antibacterial substances produced by those isolates were degraded by all proteolytic enzymes tested (protease type XIV, proteinase K, and α-chymotrypsin), indicating their proteinaceous nature (bacteriocin), as presented in Table 2.6. The isolates were subjected to *16S rRNA* gene sequencing and identified as *Streptococcus uberis* FT86, *S. uberis* FT126, *S. uberis* FT190 and *L. paraplantarum* FT259.

Table 3.6 Evaluation of the proteinaceous nature of antimicrobial substances (as described by Lewus, Kaiser and Montville, 1991) produced by selected isolates

Isolate	Source	Degradation of antibacterial compound			
		Protease type XIV	Proteinase K	α-chymotrypsin	Water*
***Streptococcus uberis* FT86**	Cow milk	+	+	+	-
***Streptococcus uberis* FT126**	Cow milk	+	+	+	-
***Streptococcus uberis* FT190**	Cow milk	+	+	+	-
***Lactobacillus paraplantarum* FT259**	Cow cheese	+	+	+	-

* Negative control; (+) : disappearance of the inhibition zone, which indicated that the substance was degraded by the proteolytic enzyme; (-) : no change in the inhibition zone, which indicated that the substance was not degraded by the proteolytic enzyme

Figure 3.4 illustrates the antibacterial activity of *Streptococcus uberis* FT86 against *L. monocytogenes* IAL 633. No halos of inhibition were detected after action of proteolytic enzymes, indicating the proteinaceous nature of the antimicrobial substance produced.

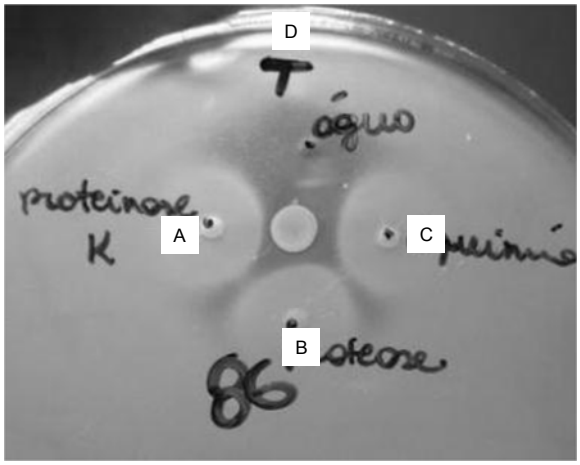


Figure 3.4 Photography of an agar plate (spot-on-the-lawn) that illustrates the susceptibility of the antibacterial compound to three proteolytic enzymes: proteinase K (A), protease type XIV (B) and α-chymotrypsin (C). *Streptococcus uberis* FT86 was the producer strain while indicator strain was Listeria monocytogenes IAL 633. Water (D) was used as negative control.

S. uberis isolates belonged to the same species in spite of being isolated from cow milk obtained from different animals. Further analyses are necessary to verify their genetic similarity.

The antibacterial spectra of bacteriocinogenic isolates shown in Table 3.7 reveal isolates that were inhibitory against *Listeria* species and LAB. The strain *S. uberis* FT190 presented the strongest antilisterial activity in comparison to the other strains, and *S. uberis* FT126 was not inhibitory to *L*. *monocytogenes* ATCC 19115. *L. paraplantarum* FT259 was the only strain that did not inhibit *Carnobacterium maltaromaticum* A9b-. However, no inhibitory activity was detected against Gram-negative bacteria nor staphylococci, by none of the strains.

Table 3.7 Antibacterial spectra of *S. uberis* FT86, *S. uberis* FT126 and *S. uberis* FT190 (isolated from cow milk, in MRS agar) and *L. paraplantarum* FT259 (isolated from cow cheese in MRS agar). All the isolates were incubated at 25 °C for 48 h. The values show the diameter of the inhibition halo obtained on agar plates, in mm*

Indicator bacteria	**Antibacterial activity – diameter (mm)**			
	Isolates			
	***S. uberis* FT86**	***S. uberis* FT 126**	***S. uberis* FT 190**	***L. paraplantarum* FT259**
Gram-positive				
Carnobacterium maltaromaticum A9b-	17	14	13	-
Lactobacillus sakei ATCC 15521	15	9	10	8
Listeria innocua ATCC 3309	22	20	25	13
Listeria monocytogenes IAL 633	23	12	20	12
Listeria monocytogenes ATCC 19115	26	-	30	17
Staphylococcus aureus ATCC 29213	-	-	-	-
Staphylococcus epidermidis ATCC 14990	-	-	-	-
Gram-negative				
Cronobacter sakazakii ATCC 29004	-	-	-	-
Enterobacter aerogenes CDC1535	-	-	-	-
Escherichia coli CDC 02A.2B	-	-	-	-
Klebsiella pneumonia ATCC 10031	-	-	-	-
Proteus mirabilis CDC 305	-	-	-	-
Pseudomonas aeruginosa ATCC 14502	-	-	-	-
Salmonella enterica subsp. *enterica* serovar Enteritidis ATCC 13076	-	-	-	-

*According to Lewus, Kaiser and Montville (1991). *S.: Streptococcus, L.: Lactobacillus*

Dairy products are important sources for the isolation of bacteriocinogenic LAB. In 2000, Rodríguez et al. obtained 1340 LAB isolates from raw cow milk, among which 321 were bacteriocinogenic strains with inhibitory activities against *L. monocytogenes*, *Staphylococcus aureus* and/or *Clostridium tyrobutyricum*. Those authors detected among the bacteriocins produced by the LAB strains, enterocin AS-48, nisin and lacticin 481. In the present study, however, the bacteriocinogenic strains did not show antagonistic activity against staphylococci.

Bacteriocinogenic enterococci are frequently isolated from raw milk samples. Nero et al. (2009) evaluated the occurrence of autochthonous bacteria in raw cow milk, with activity against *L. monocytogenes* and *Salmonella enterica* subsp. *enterica* serovar Enteritidis. Most of the isolates were identified as *Lactococcus lactis* subsp. *lactis* and *Enterococcus faecium*. Similarly, Chanos and Williams (2011) analyzed 40 samples of raw sheep milk using MRS, M17 and bile esculin agar. In that paper, 332 bacterial isolates were obtained, and 17 were able to inhibit *L. monocytogenes*. All the isolates with antagonistic activity were identified as *E. faecium*. In the present work, the four more antagonistic strains did not belong to the genera *Lactococcus* or *Enterococcus*. This may be due to the number of samples analyzed, difference in milk microbiota or even to a diverse indicator microorganism used to detect bacteriocin production.

Cheese sample also represent an important source of bacteriocinogenic strains. In 1997, Coogan et al. analyzed 389 samples of cheese and other fermented products and obtained 4379 isolates. Out of those isolates, 2257 were tested for antimicrobial activity, and 11 % produced bacteriocins. Among the four bacteriocinogenic strains obtained in this work, only one was isolated from cheese (*L. paraplantarum* FT259).

In the present study, three bacteriocinogenic strains of *S. uberis* and one *L. paraplantarum* were isolated and showed inhibitory activity toward *Lactobacillus sakei, L. monocytogenes* and *Listeria innocua*, whereas *S. aureus*, *Staphylococcus epidermidis* and all Gram-negative bacteria tested were not inhibited. These results are in agreement with those found by Stevens et al. (1991) that reported Gram-negative bacteria are not usually inhibited by bacteriocins from LAB, due to the protection of the cell membrane by the outer membrane.

Streptococcus uberis is one of the main agents causing clinical mastitis worldwide (LEIGH, 1999), and there are few studies on bacteriocins produced by this species. The strain *S. uberis* 42 was characterized elsewhere by the production of nisin U and uberolisin, a cyclic bacteriocin similar to enterocin AS-48 (WIRAWAN et al., 2006; WIRAWAN et al., 2007). In 2007, Heng et al. described ubericin A, a novel class IIa bacteriocin described for the genus *Streptococcus*.

The species *L. paraplantarum* was first described in 1996 by Curk, Hubert and Bringel, who observed some strains of lactobacilli had characteristics similar to *L. plantarum*, but they did not catabolize α-methyl-D-mannoside. Moreover, strains

when analyzed by RFLP (restriction fragment length polymorphism) techniques had different restriction profiles in comparison with *L. plantarum* and *L. pentosus*, suggesting their allocation into a new species. *L. paraplantarum* is a facultatively heterofermentative rod-shaped Gram-positive bacterium that grows from 15 °C to 37 °C, with NaCl concentrations up to 8 % and it is closely related to *L. plantarum* and *L. pentosus* (CURK; HUBERT; BRINGEL, 1996). Until now, only one bacteriocin produced by *L. paraplantarum* has been characterized: the paraplantaracin C7, with a molecular mass of 3,800 Da (LEE et al., 2007). Also, to our knowledge, there is only one report on the probiotic potential of a *L. paraplantarum* strain (NIKOLIC et al., 2012).

3.1.2 Evaluation of antifungal activity

Antifungal activity was detected in 198 isolates (24.3 %) initially screened for antifungal activity, as described in item 2.5. The most active strains identified as *Weissella confusa* FT424, *Weissella hellenica* FT476, *Leuconostoc citreum* FT671 and *L. plantarum* FT723 (identified according to Item 2.7) were selected for further assays. Other strains with less antifungal activity are also presented in Table 3.5. During the confirmation of the antifungal activity in modified MRS agar (Item 2.5.1), *W. confusa* FT424 and *L. plantarum* FT723 inhibited the growth of *P. expansum* UBOCC 1.08.102 (Figures 3.5 and 3.6). Inhibition of *Y. lipolytica* UBOCC 2.11.004 was not detected by any of the strains. The initial pH of the medium without LAB strains was ~5.6, but after bacterial growth, the final pH of the medium dropped to ~4.4, indicating the production of organic acids. In the fermented milk assay (Item 2.5.2), only *L. plantarum* FT723 inhibited *P. expansum* UBOCC 1.08.102 after two weeks at 10 °C (Figure 3.7). Similarly to the agar assay, no inhibition of *Y. lipolytica* UBOCC 2.11.004 was detected.

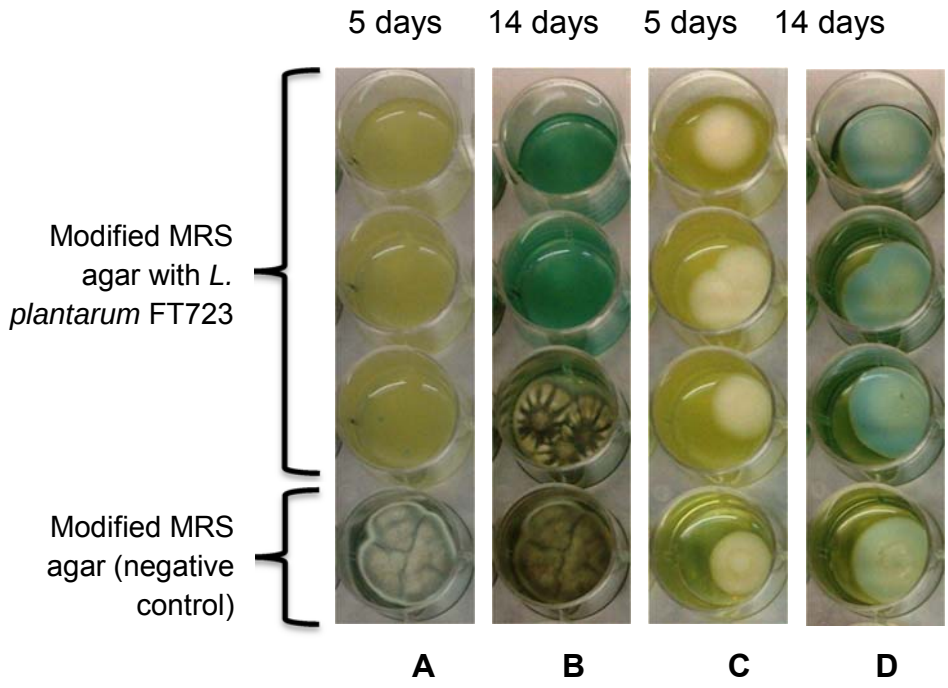


Figure 3.5 Results for test of inhibition of *Penicillium expansum* (A and B) and *Yarrowia lipolytica* (C and D) by *Lactobacillus plantarum* FT723 in modified MRS agar after 5 and 14 days of incubation at 25 °C. (A) and (B) were inoculated with 100 spores per well of *P. expansum*; (C) and (D) were inoculated with 100 CFU per well of *Y. lipolytica*. Growth inhibition or reduction on colony size of *P. expansum* or *Y. lipolytica* was indicative of LAB antifungal activity. Tests were done in triplicates in the same plate, as described in item 2.5.1.

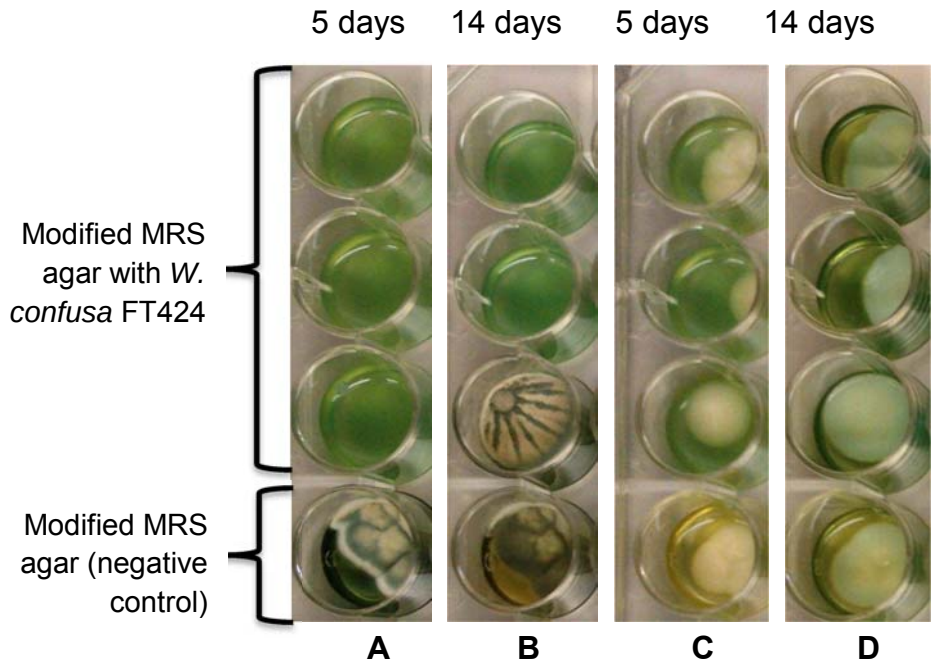


Figure 3.6 Results for test of inhibition of *Penicillium expansum* (A and B) and *Yarrowia lipolytica* (C and D) by *Weissella confusa* FT424 in modified MRS agar after 5 and 14 days of incubation at 25 °C. (A) and (B) were inoculated with 100 spores per well of *P. expansum*; (C) and (D) were inoculated with 100 CFU per well of *Y. lipolytica*. Growth inhibition or reduction on colony size of *P. expansum* or *Y. lipolytica* was indicative of LAB antifungal activity. Tests were done in triplicates in the same plate, as described in item 2.5.1.

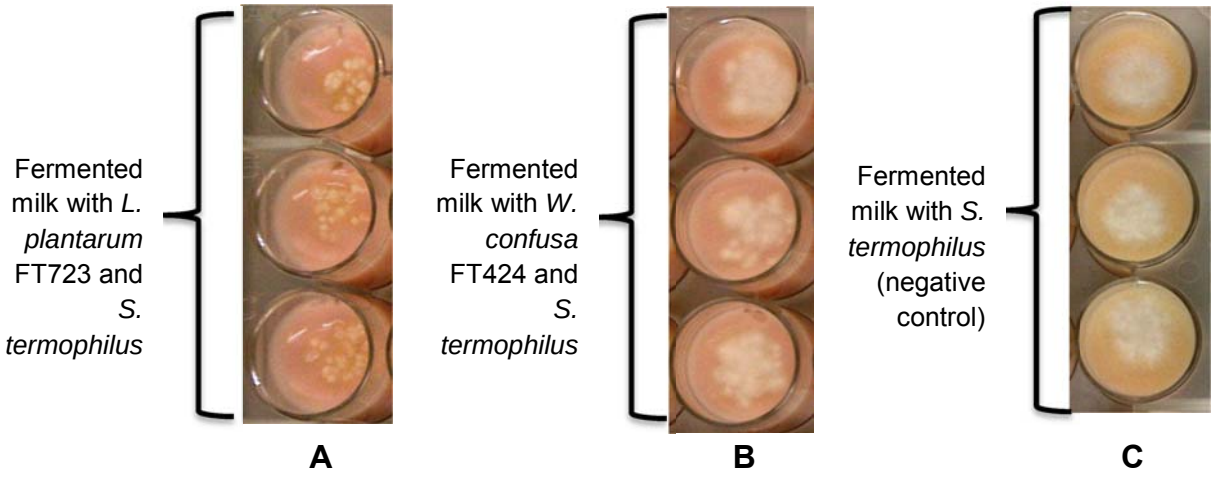


Figure 3.7 Results for test of inhibition of *Penicillium expansum* by *Lactobacillus plantarum* FT723 (A) and *Weissella confusa* FT424 (B) in fermented milk model (with *Streptococcus thermophilus* as starter strain), after 14 days of incubation. Fermented milk with only *S. thermophilus* was used as negative control (C). After 24 h of incubation (production of fermented milk), wells were inoculated with 100 spores of *P. expansum* and incubated at 10 °C. Tests were done in triplicates in the same plate, as described in item 2.5.2.

In 2010, Voulgari et al. analyzed 81 isolates of LAB from cheese and other dairy products and observed 31 isolates (38 %) presented anti-mould (*Penicillium* spp.) and anti-yeast (*Debaryomyces hansenii* and *Saccharomyces cerevisiae*) activities on MRS agar. In our study, many isolates presented antifungal activity against *K. lactis* (presumptive result was 24.3 % of isolates) on MRS agar without sodium acetate and were identified as *Weissella* spp., *Leuconostoc* spp. and *Lactobacillus* spp. The strains most inhibitory towards *P. expansum* in modified MRS agar were identified as *L. plantarum* FT723 and *W. confusa* FT424. In a similar study, Baek et al. (2012) evaluated the antifungal activity of *L. citreum* and *W. confusa* in rice cakes, reporting both strains presented inhibitory activity against *Penicillium crustosum*, attributed to the production of organic acids. In our study, production of organic acids by *L. plantarum* FT723 and *W. confusa* FT424 was unlike to be the main mechanism of inhibition of *P. expansum*, since it is highly resistant to organic acids (DELAVENNE et al., 2013). Concerning food safety aspects, *L. plantarum* has a GRAS status (FOOD AND DRUG ADMINISTRATION, 2012) and is a good candidate for use in biopreservation systems. *Weissella* spp. are non-spore-forming, Gram-positive, catalase negative bacteria that are present in several environments such as fermented food, sugar cane, and gastrointestinal tract of human and animals (LEE et al., 2012). However, until this date, scarce data exists on the safety aspects of this microorganism.

3.1.3 Evaluation of proteolytic activity

After the preliminary assessment of proteolytic activity, 123 isolates (60 Gram-positive cocci and 63 Gram-positive rods) were selected for confirmation of proteolysis in skim milk followed by analysis by SDS-PAGE. One isolate from cow milk (*Enterococcus faecalis* FT132), one from buffalo milk (*E. faecalis* FT522) and one from goat cheese (*Lactobacillus paracasei* FT700) were found to be most proteolytic strains, as illustrated in Figure 3.8. The degradation of α-casein, β-casein, β-lactoglobulin and α-lactalbumin was confirmed by comparison with the control (skim milk). Similarly, in 2011, Ahmadova et al. isolated 147 strains from traditional Azerbaijani dairy products, but only six strains, also identified as *E. faecalis,* showed intense proteolytic activity. El-Ghaish et al. (2010) evaluated the proteolytic activity of 151 LAB (cocci) on skim milk agar and cultivation in skim milk followed by SDS-

PAGE analysis. It was observed that 24 isolates presented hydrolysis halos on skim milk agar, but only six isolates were confirmed as proteolytic, all identified as *E. faecalis* (n = 4) and *E. faecium* (n = 2). Those data show the importance of confirming the proteolytic activity by polyacrylamide gel electrophoresis. The present study revealed the proteolytic capacity of *E. faecalis* species, as well as the *L. paracasei* strain isolated from goat cheese.

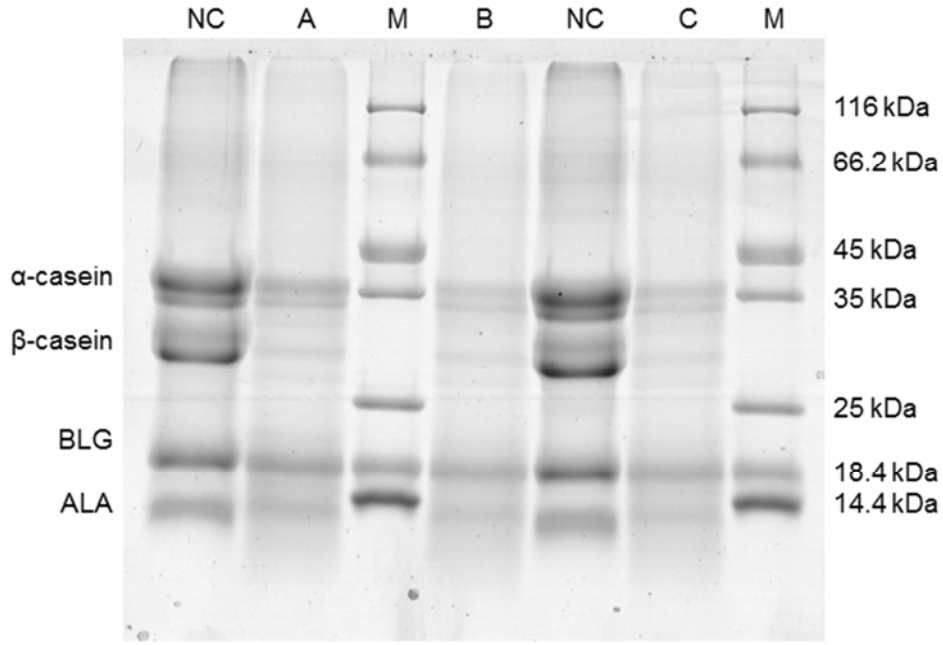


Figure 3.8 Photography of a SDS-PAGE gel from samples obtained after the hydrolysis of skim milk by selected bacteria. NC: negative control (skim milk); A: *Enterococcus faecalis* FT132; B: *Enterococcus faecalis* FT522; C: *Lactobacillus paracasei* FT700; BLG: β-lactoglobulin; ALA: α-lactalbumin.

E. faecalis is a facultatively anaerobic bacterium that initially colonizes the infant gastrointestinal tract (GIT) and that is also prevalent in the adult gastrointestinal tract (CUIV et al., 2013). Despite its implication in sporadic cases of diseases such as urinary tract infection, endocarditis, peritonitis, bacteremia, and wound infection, *E. faecalis* has proven to be potentially beneficial to humans, since many strains have been used as food-starters and probiotics. One example is the *E. faecalis* Symbioflor® 1, which has been successfully used as probiotic for more than 50 years (FRITZENWANKER et al., 2013).

L. paracasei is a facultatively heterofermentative member of the genus *Lactobacillus*, which comprises more than 130 species. The "*Lactobacillus casei*

group" (*L. casei*, *L. paracasei* and *Lactobacillus rhamnosus*) comprises several well-known probiotic-marketed strains and may present probiotic traits such as acid and bile salts tolerance, which are important for survival of the bacteria in the GIT (DOUILLARD et al., 2013; FELIS; DELLAGLIO, 2007; TOH et al., 2013).

4. Conclusions

LAB strains were isolated from milk and cheese, selected for antimicrobial and proteolytic activities. In summary, *Streptococcus uberis* FT86, *S. uberis* FT126, *S. uberis* 190 and *Lactobacillus paraplantarum* FT259 were characterized as bacteriocinogenic strains; *L. plantarum* FT723 and *Weissella confusa* FT424 presented antifungal activity; *Enterococcus faecalis* FT132, *E faecalis* FT522 and *L. paracasei* FT700 were identified as proteolytic bacteria. Strains with these traits may be of technological interest for dairy industry offering alternatives for improving safety, shelf life or design of fermented products. In the next chapters, the evaluation of functional properties of selected bacteria will be presented.

4. Evaluation of the proteolytic activity of *Enterococcus faecalis* FT132 and *Lactobacillus paracasei* FT700, isolated from dairy products, using milk proteins as substrates

Evaluation of the proteolytic activity of *Enterococcus faecalis* FT132 and *Lactobacillus paracasei* FT700, isolated from dairy products, using milk proteins as substrates

Abstract

Lactic acid bacteria (LAB) have been used by mankind from immemorial times due to their technological properties and ability to improve sensorial properties of foods. Some of LAB strains are also able to hydrolyze proteins, which increases their digestibility, and contributes to the production of desirable flavors. This study aimed to evaluate the proteolytic activity of two LAB strains isolated from Brazilian milk and cheese. The proteolytic activity of *Enterococcus faecalis* FT132 and *Lactobacillus paracasei* FT700 was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and high-performance liquid chromatography (HPLC) using milk proteins as substrates in different conditions. Both *E. faecalis* FT132 and *L. paracasei* FT700 showed proteolytic activities at pH 6.5, in the range of 37 to 42 °C. Their proteolytic activities detected were due to metalloproteases. Hydrolyzed proteins in the supernatant of fermented milk obtained by the action of both strains did not show any inhibitory activity against *Listeria monocytogenes*. Concerning microbial safety, lactobacilli have "generally recognized as safe (GRAS) status", contrary to enterococci. In this study, it was determined that *E. faecalis* FT132 harbored three virulence genes *asa*1, *ace* and *gel*E and it was resistant to erythromycin and tetracycline, indicating that this strain may not be added to food products. However, the peptides produced after hydrolytic activity on milk proteins by both strains may have some biological activity, as evaluated in chapter 5.

Key-words: lactic acid bacteria, milk, proteolysis, *Enterococcus*, *Lactobacillus*.

Avaliação da atividade proteolítica de *Enterococcus faecalis* FT132 e *Lactobacillus paracasei* FT700, isolados a partir de produtos lácteos, utilizando proteínas de leite como substratos

Resumo

As bactérias láticas (BAL) têm sido utilizadas pela humanidade desde tempos remotos, devido às suas propriedades tecnológicas e capacidade de melhorar as propriedades sensoriais dos alimentos. Algumas BAL também são capazes de hidrolisar proteínas, o que aumenta a sua digestibilidade, e contribui para a produção de sabores agradáveis nos alimentos. Este estudo teve como objetivo avaliar a atividade proteolítica de duas linhagens de BAL isoladas de leite e queijo produzidos no Brasil. A atividade proteolítica de *Enterococcus faecalis* FT132 e *Lactobacillus paracasei* FT700 foi avaliada utilizando proteínas do leite como substratos, em diferentes condições, sendo analisada por eletroforese em gel de poliacrilamida com dodecil sulfato de sódio (*sodium dodecyl sulfate polyacrylamide gel electrophoresis*, SDS-PAGE) e cromatografia líquida de alta eficiência (*high-performance liquid chromatography*, HPLC). Ambos *E. faecalis* FT132 e *L. paracasei* FT700 apresentaram atividades proteolíticas em pH 6,5 e entre 37 e 42 °C. Foi detectada a atividade proteolítica devido à metaloproteases em ambas as linhagens. Hidrolisados de proteínas presentes no sobrenadante de leite fermentado, obtidos pela ação de ambas as linhagens, não mostraram qualquer atividade inibitória contra *Listeria monocytogenes*. Com relação à segurança microbiológica, os lactobacilos são geralmente reconhecidos como seguros (*generally recognized as safe*, GRAS), o que não se aplica aos enterococos. Neste estudo detectou-se que *E. faecalis* FT132 possui os genes *asa*1, *as* e *gel*E, e apresenta resistência a eritromicina e tetraciclina, o que indica que esta linhagem não pode ser adicionada a alimentos. No entanto, os peptídeos produzidos após a atividade hidrolítica sobre proteínas do leite podem apresentar atividade biológica, tal como avaliado no capítulo 4.

Palavras-chave: bactérias lácticas, leite, proteólise, *Enterococcus*, *Lactobacillus*.

Evaluation de l'activité protéolytique d'*Enterococcus faecalis* FT132 et de *Lactobacillus paracasei* FT700, isolés à partir de produits laitiers, en utilisant les protéines du lait comme substrats

Résumé

Les bactéries lactiques (BAL) ont été utilisées par l'humanité depuis des temps immémoriaux en raison de leurs propriétés technologiques et de leur capacité à améliorer les propriétés sensorielles des aliments. Certaines souches de BAL sont également capables d'hydrolyser les protéines laitières, ce qui augmente leur digestibilité, et contribue à la productiond'arômes. Cette étude visait à évaluer l'activité protéolytique de deux souches de BAL isolées à partir d'échantillons de lait et de fromage provenant sud est du Brésil. Les activités protéolytiques d' *Enterococcus faecalis* FT132 et de *Lactobacillus paracasei* FT700 ont été confirmées en utilisant des protéines laitières comme substrats dans différentes conditions d'incubation, suivie d'une électrophorèse sur gel de polyacrylamide en conditions dénaturantes (*sodium dodecyl sulfate polyacrylamide gel electrophoresis*, SDS-PAGE) et d'une chromatographie liquide à haute performance (*high-performance liquid chromatography*, HPLC). Les deux souches, *E. faecalis* FT132 et *L. paracasei* FT700 ont montré des activités protéolytiques à un pH de 6.5, dans un intervalle de température allant de 37 à 42 °C. Leurs activités protéolytiques étaient dues à des métallo-protéases. Les hydrolysats de protéines laitières obtenus dans les surnageants de laits fermentés par les deux souches, n'ont montré aucune activité inhibitrice contre *Listeria monocytogenes*. En ce qui concerne l'innocuité des souches, la plupart des lactobacilles possède le *status* "*generally recognized as safe* (GRAS)", en revanche, ce n'est pas le cas des entérocoques. Dans cette étude, il a été détecté que *E. faecalis* FT132 possédait les gènes *asa*1, *as* et *gel*E, et qu'il était résistant à l'érythromycine et à la tétracycline, ce qui indique que cette souche ne peut pas être ajoutée aux aliments. Cependant, les peptides produits suite aux activités hydrolytiques de ces souches sur les protéines laitières pourraient avoir une certaine activité biologique, comme je l'ai montré au chapitre 5.

Mots-clés : bactéries lactiques, le lait, la protéolyse, *Enterococcus*, *Lactobacillus*.

1. Introduction

Lactic acid bacteria (LAB) are widely used as starters in fermented dairy products, mainly *Streptococcus thermophilus*, *Lactococcus lactis*, *Lactobacillus helveticus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, in which the proteolytic system plays a main role (SAVIJOKI; INGMER; VARMANEN, 2006). Together, the non-starter LAB (NSLAB) are very important in cheese ripening, such as *Enterococcus durans*, *Enterococcus faecalis*, *Enterococcus faecium*, *Lactobacillus brevis*, *Lactobacillus casei*, *Lactobacillus curvatus*, *Lactobacillus fermentum*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Pediococcus acidilactici*, *Pediococcus pentosaceus* and other LAB (SETTANNI; MOSCHETTI, 2010). During the process of milk fermentation, LAB hydrolyze milk proteins, thereby releasing bioactive peptides. The characteristics of this process are important to scale up the fermentation to industrial levels (KORHONEN, 2009; TZVETKOVA et al., 2007). Also, according to Potier and Tomé (2008), casein and whey hydrolysates have increased digestibility in comparison to native milk protein, which is very important in clinical diets for pathologies with reduced absorptive capacity and food allergies. In this study, two NSLAB isolated from dairy products (*Enterococcus faecalis* FT132 and *Lactobacillus paracasei* FT700) were evaluated concerning their proteolytic activity in different conditions and substrates, with the aim of future application in the production of fermented dairy products or hydrolyzed milk protein concentrates.

2. Materials and methods

2.1 Strains and culture conditions

E. faecalis FT132 and *L. paracasei* FT700 were isolated from Brazilian cow milk and goat cheese, respectively. They were screened for proteolytic activities and identified by molecular tests, as presented in chapter 3. Both strains were routinely cultivated in MRS broth (De Man, Rogosa, Sharpe broth, AES Laboratory, France) at 37 °C for 24 h and stored in MRS broth with 20 % w/v glycerol (Synth, Brazil) at -80 °C.

2.2 Evaluation of the proteolytic activity

2.2.1 Proteolysis of milk proteins

A 24 h culture of LAB incubated at 37 °C was washed with 0.85 % w/v NaCl solution and added to skim milk (Délisse UHT, France) to obtain a final concentration of *ca.* 1 × 10^7 CFU/mL. The inoculated milk was incubated for 24 h at 37 °C, homogenized and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as described below.

2.2.2 Proteolytic activity at different pH and temperatures

A 24 h culture of LAB, incubated at 37 °C was washed with 0.85 % w/v NaCl solution and added to skim milk to obtain a concentration of *ca*. 1 × 10^7 CFU/mL. The inoculated milk was incubated for 24 h at 37 °C, homogenized and used to inoculate MRS broth at a concentration of *ca*. 1 × 10^7 CFU/mL. After 24 h at 37 °C, two hundred microliters were used to seed Milk Citrate Agar (MCA) plates, containing 44 % v/v skim milk (Délisse), 0.8 % w/v sodium citrate, 0.1 % w/v yeast extract, 0.5 % w/v glucose and 1.5 % w/v agar (FIRA et al., 2001), and incubated for 24 h at 37 °C. Colonies were collected from the MCA plates, washed twice with 0.85 % w/v NaCl + 10 mM $CaCl_2$ solution and adjusted to OD_{600nm} ~20 with the same solution. The substrate solutions (Na-caseinate, whey, and β-lactoglobulin) were added 1:1 to the cell suspension to obtain 1 mL of final volume and incubated at 37 °C for 24 h.

The substrate solutions were prepared at 12 mg/mL for Na-caseinate (obtained on laboratory scale, by precipitation at pH 4.6) and at 6 mg/mL for whey proteins (obtained on laboratory scale, by precipitation at pH 4.6) in 100 mM of phosphate buffer, at pH 6.0, 6.5 and 7.0. The whey solution was heated at 80 °C for 30 min before use. After preliminary studies, the pH 6.5 was chosen for all experiments, based on the best pH for the proteolytic activity of selected LAB. The same procedure was repeated with substrate solutions (pH 6.5) incubated at 30, 37 and 42 °C. The hydrolyzed samples were analyzed by SDS-PAGE, and LAB proteolytic activity was evaluated by visual comparison with the intensity of the bands on the gels.

2.2.3 Effect of protease inhibitors on the proteolytic activity of LAB

A 24 h culture of LAB incubated at 37 °C was washed with a 0.85 % w/v NaCl solution and added to skim milk to obtain a final concentration of *ca*. 1×10^7 CFU/mL. The inoculated milk was incubated for 24 h at 37 °C, homogenized and used to inoculate MRS broth at a concentration of *ca*. 1×10^7 CFU/mL. After incubation for 24 h at 37 °C, two hundred microliters of fermented milk were used to seed MCA plates which were incubated for 24 h at 37 °C. Colonies were collected, washed twice with 0.85 % w/v NaCl and 10 mM $CaCl_2$ solution, and the bacterial suspension was adjusted to OD_{600nm} ~40. The protease inhibitor solution (EDTA, iodoacetic acid or Pefabloc®, Sigma-Aldrich, USA) was added 1:1 to the cell suspension to obtain 1 mL of final volume, and incubated at 37 °C for 90 min. Next, the different substrate solutions (Na-caseinate and whey, prepared as described previously) were added to these pre-incubated bacterial suspensions (1:1) and incubated at 37 °C for 24 h.

2.2.4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Samples containing hydrolyzed proteins were analyzed in a vertical electrophoresis system (Mini-PROTEAN II Electrophoresis Cell, Bio-rad Laboratories, USA) according to El-Ghaish et al. (2010), using polyacrylamide gel (12 % resolving gel and 3.2 % stacking gel, Table 4.1). The sample buffer contained 4 % w/v SDS (sodium dodecyl sulfate, Synth), 3 % v/v mercaptoethanol (Sigma-Aldrich), 10 % v/v glycerol (Synth), 50 mM tris-HCl pH 6.8 (Serva, Germany) and 0.07 % w/v bromophenol blue (Bio-Agency, Brazil). Samples were heated at 100 °C for 3 min and analyzed by SDS-PAGE. The running buffer was prepared with 50 mM tris base (Serva), 0.384 M glycine (Sigma-Aldrich) and 0.1 % w/v SDS. The electrophoretic separation was carried out at 10 mA for the stacking gel and 20 mA for the resolving gel, followed by staining with an aqueous solution containing 0.2 % w/v Coomassie Brilliant Blue R-250 (Sigma-Aldrich), 30 % v/v ethanol (Synth) and 5 % v/v glacial acetic acid (Synth). The gels were destained with an aqueous solution containing 30 % v/v ethanol and 5 % v/v glacial acetic acid, and photographed (MiniBis UV, DNA Bio-Imagings Systems, Israel).

Table 4.1 Composition of polyacrylamide gels used for SDS-PAGE analyses of hydrolyzed milk proteins. Values are presented for plates with 0.75 mm spacers

Component	Staking gel (3.2 %)	Resolving gel (12 %)
Acrylamide-bis[1] (29:1) 40 %	200 µL	2400 µL
Tris-HCl[2] 2 M, pH 8.8	0	1330 µL
Tris-HCl[2] 0.5 M, pH 6.8	300 µL	0
Water	2000 µL	4270 µL
SDS[3] 10 %	25 µL	80 µL
TEMED[4]	4 µL	16 µL
Ammonium persulfate [2] 10 % (aqueous solution)	20 µL	80 µL
Total	**2549 µL**	**8176 µL**

[1] Supplier: Sigma-Aldrich, USA.
[2] Supplier: Synth, Brazil.
[3] Sodium dodecyl sulfate (Synth).
[4] N,N,N′,N′-Tetramethylethane-1,2-diamine (Sigma-Aldrich).

2.2.5 High-performance liquid chromatography (HPLC) analysis of hydrolyzed proteins

HPLC analyses were performed using a C_{18} column (5 µm, Symmetry 300 C_{18}, Waters, USA) in a Waters HPLC System Alliance. Chromatographic separation was conducted using water / acetonitrile (Biosolve, Netherlands) / trifluoroacetic acid (95:5:0.05) as solution A, and isopropyl alcohol (Sigma-Aldrich) / acetonitrile / trifluoroacetic acid (20:80:0.03) as solution B. The flow rate was 1.2 mL/min and the peptides were detected at 220 nm in a Waters 996 photodiode array detector.

2.3 Virulence genes and antibiotic resistance for *E. faecalis* FT132

As other LAB, *L. paracasei* FT700 is intrinsically resistant to some antimicrobial agents such as vancomycin and ciprofloxacin (NELSON et al., 1999; HUMMEL et al., 2007), as demonstrated by antibiotic susceptibility tests in chapter 6. However, as *Enterococcus* strains may possess mobile antibiotic resistance genes transferable to other strains, as well as virulence factors, it is important to check the presence of such traits (LEE; LEE; LEE, 2008). Polymerase chain reaction (PCR)

was used to search for virulence genes in *E. faecalis* FT132 such as *ace* (adhesin of collagen protein), *asa*1 (aggregation substance), *cyl*A (cytolysins), *efa*A (cell wall adhesin), *esp* (enterococcal surface protein) and *gel*E (gelatinase). For this purpose, *E. faecalis* FT132 genomic DNA was extracted (Omega Bio-Tek kit, USA) and 40 ng were used as template for amplifications with 0.2 µM of each primer and 4.4 µL of 5x BioAmp (BioFidal, France) master mix (final volume 25 µL). The thermal cycling used was: initial denaturation step at 94 °C for 5 min, following denaturation steps at 94 °C for 1 min, annealing for 1 min at appropriate temperature depending on tested primers (Table 4.2), extension at 72 °C for 1 min, and final extension at 72°C for 5 min (total of 35 cycles). PCR products were analyzed by electrophoresis at 100 V for 30 minutes using 1 % w/v agarose gel (containing 0.5 µg/mL ethidium bromide) in TAE buffer. Next, the bands were visualized under UV light (Gel Doc^{TM} XR, Bio-rad Laboratories).

Table 4.2 Oligonucleotide sequences of the primers used in this study to detect virulence genes in *Enterococcus faecalis* FT132

Target genes	Oligonucleotide sequences (5'-3')	Annealing temperature (°C)	Reference
ace	Fw: GAA TTG AGC AAA AGT TCA ATC G Rv: GTC TGT CTT TTC ACT TGT TTC	56	OMAR et al., 2004
*asa*1	Fw: GCA CGC TAT TAC GAA CTA TGA Rv: TAA GAA AGA ACA TCA CCA CGA	56	VANKERCKHOVEN et al., 2004
*cyl*A	Fw: ACT CGG GGA TTG ATA GGC Rv: GCT GCT AAA GCT GCG CTT	56	VANKERCKHOVEN et al., 2004
*efa*A	Fw: GAC AGA CCC TCA CGA ATA Rv: AGT TCA TCA TGC TGT AGT A	54	EATON; GASSON, 2001
esp	Fw: AGA TTT CAT CTT TGA TTC TTG G Rv: AAT TGA TTC TTT AGC ATC TGG	56	VANKERCKHOVEN et al., 2004
*gel*E	Fw: TAT GAC AAT GCT TTT TGG GAT Rv: AGA TGC ACC CGA AAT AAT ATA	56	VANKERCKHOVEN et al., 2004

Antibiotic resistance in *E. faecalis* FT132 was evaluated using the Kirby-Bauer disk diffusion susceptibility test (BAUER; KIRBY, 1966), as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2012) with ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), penicillin G (10 U), rifampicin (5 µg), tetracycline (30 µg) and vancomycin (30 µg). Antibiotic disks were purchased from Inlab, Brazil. Results were interpreted according to the cut-off level proposed for *Enterococcus* spp. by the CLSI (2012), with strains considered

resistant if inhibition zone diameters were smaller than 16 mm for ampicillin, 12 mm for chloramphenicol, 15 mm for ciprofloxacin, 13 mm for erythromycin, 14 mm for penicillin G, 16 mm for rifampicin, 14 mm for tetracycline and 14 mm for vancomycin.

2.4 Preparation of fermented milk supernatant (FMS) containing hydrolyzed milk proteins

A 24 h culture of LAB in MRS incubated at 37 °C was washed with 0.85 % w/v NaCl and added to sterile skim milk to obtain a final concentration of *ca*. 1×10^7 CFU/mL. The inoculated milk was incubated for 24 h at 37 °C and centrifuged at 8000 × *g* for 20 minutes at 4 °C. The supernatant was collected, filtered through a 0.8 µm filter (Minisart, Sartorius Stedim Biotech, Germany), and desalted by using a C_8 cartridge (Sep-Pak Vac 20 cc 5 g, Waters, Ireland). The supernatant was loaded onto the cartridge, which was previously conditioned with 50 % v/v acetonitrile and equilibrated with the solution A (aqueous solution with 0.03 % v/v trifluoroacetic acid, TFA, Sigma-Aldrich). After a washing step with solution A, hydrolyzed milk proteins were eluted with solution B (80 % v/v acetonitrile, 20 % v/v isopropyl alcohol and 0.03 % v/v TFA, JT Baker, USA). The desalted supernatant was freeze-dried and used in cell culture tests (chapter 5). Non-hydrolyzed milk proteins were obtained in the same way from sterilized skim milk added of 4.3 g/L of lactic acid (Sigma-Aldrich), because this is the average lactic acid concentration produced in fermented milk by both strains, as measured according to instructions of manufacturer using the D/L Lactic Acid UV-Method Kit (Roche, Germany). This control with non-hydrolyzed proteins was called “acidified milk supernatant” (AMS).

2.5 Antimicrobial activity of fermented milk supernatant (FMS)

FMS obtained from *E. faecalis* FT132 and *L. paracasei* FT700, as well as the control AMS, were resuspended in water at 10 mg/mL and tested with regard to antimicrobial activity against *Listeria monocytogenes* IAL 633 by the agar overlay method. For this, ten microliters of each sample were applied in BHI agar plates (Brain Heart Infusion broth plus 1.5 % w/v bacteriological agar, Oxoid, UK) that were previously inoculated with 10 mL of soft BHI agar (BHI broth plus 0.8 % w/v bacteriological agar, Oxoid) inoculated with 10^7 CFU/mL of *L. monocytogenes*. Plates

were incubated at 37 °C for 24 h, and the presence of clear zones would indicate antimicrobial activity of hydrolyzed proteins.

3. Results and discussion

3.1 Evaluation of the proteolytic activity

In this study, *E. faecalis* FT132 and *L. paracasei* FT700 were evaluated for proteolytic activities and ability to hydrolyze milk proteins. Both strains were previously isolated from Brazilian dairy products and identified by *16S rRNA* gene sequencing (chapter 3).

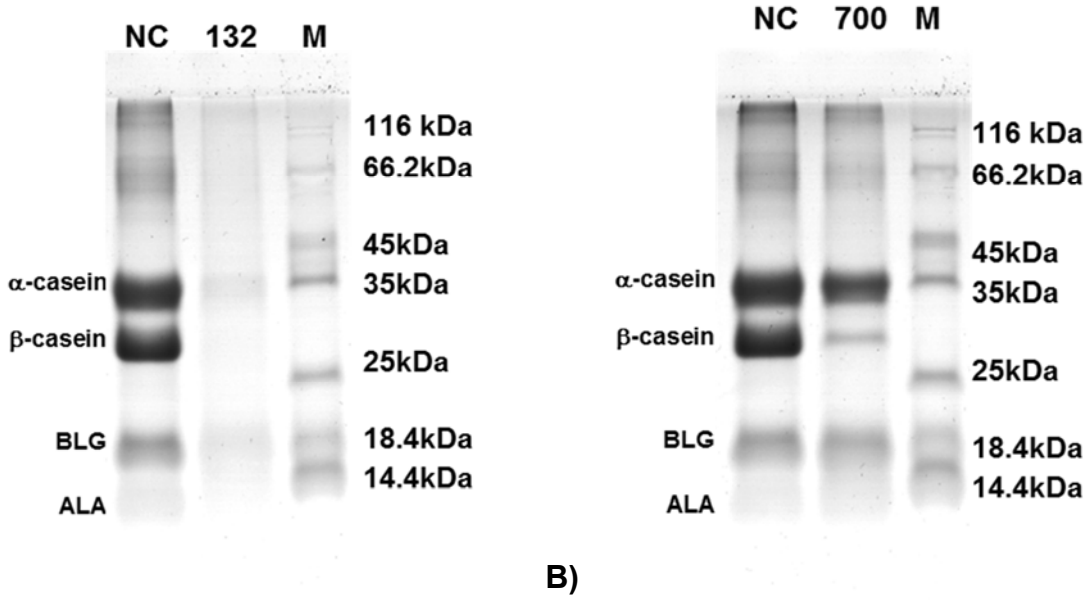
NSLAB cultures present in cheese comprise *Pediococcus* spp., *Leuconostoc* spp. and also *E. faecalis* and *L. paracasei*. These cultures contribute to proteolysis mainly due to their peptidolytic potential, increasing the amount of small peptides and free amino acids (BRIGGILER-MARCÓ et al., 2007). In 2010, Milesi et al. evaluated the impact of a *L. casei* strain in the manufacture of soft cheeses. Those authors detected an increased availability of aromatic compounds precursors, leading to improved sensory traits and positive flavor modification. Moreover, according to Milesi et al. (2009), the addition of *L. casei* and other lactobacilli in cheese manufacture may improve cheese quality the eradication of detrimental microorganisms, in addition to its potential probiotic properties.

Enterococci may naturally occur in cheeses and dairy products, but their application in food is still controversial despite the important contribution of this genus to ripening, flavor development and bacteriocin production in cheese (OGIER; SERROR, 2008). An interesting study conducted by Oumer et al. (2001) evaluated the inclusion of a bacteriocinogenic *E. faecalis* (previously evaluated concerning virulence traits) as adjunct culture during the manufacturing of Hispánico cheese. Those authors showed that the strain positively contributed to sensorial quality of Hispánico cheese throughout ripening, in comparison with the control.

3.1.1 Proteolytic activity studied on skim milk

The first step to evaluate proteolytic activity was performed by inoculation of *E. faecalis* FT132 and *L. paracasei* FT700 in skim milk. Thus, both strains were cultured

in skim milk for 24 h at 37 °C, and the fermented milk was homogenized and analyzed by SDS-PAGE, as presented in Figure 4.1.



A) **B)**

Figure 4.1 Photographies of proteolytic activity of *Enterococcus faecalis* FT132 (A) and *Lactobacillus paracasei* FT700 (B) in skim milk, analyzed by SDS-PAGE. *NC: negative control (skim milk), M: molecular weight marker, BLG: β-lactoglobulin, ALA: α-lactalbumin.

Similar results were presented in chapter 3, for the confirmation of proteolytic activity by LAB (Figure 3.8). However, those tests were performed with skim milk used for microbiology purposes (Oxoid), whereas the results presented in Figure 4.1 were obtained by the cultivation of LAB in commercial skim milk (Délisse UHT skim milk). Thus, the aim of this test was to evaluate the performance of both strains in commercial skim milk, as this product would be used in the following steps in the study. *E. faecalis* FT132 presented a higher proteolytic activity in comparison to *L. paracasei* FT700, especially on α_S-casein and BLG (β-lactoglobulin). Briggiler-Marcó et al. (2007) evaluated the proteolytic activity of a *L. casei* strain in milk and showed that it displayed a moderate proteolytic activity in comparison with other LAB strains. Still, according to Giraffa (2003), *E. faecalis* seemed to be the most proteolytic species among enterococci.

3.1.2 Effect of pH, temperature and protease inhibitors on the proteolytic activity of lactic acid bacteria

It is important to evaluate the best conditions for proteolytic activity, including temperature and pH. In addition, by using protease inhibitors, it is possible to infer the type of active proteases. EDTA, iodoacetic acid and Pefabloc® inhibit metallo-, cysteine- and serine-proteases, respectively. The reduction in the intensity of the bands in polyacrylamide gels when compared to the negative control (non-hydrolyzed proteins) was considered as positive result for protein hydrolysis, as illustrated in Figure 4.2 for the evaluation of protease inhibitors in LAB proteolytic activity. The results obtained after proteolysis of Na-caseinate and whey in all conditions tested are summarized in Tables 4.3, 4.4 and 4.5.

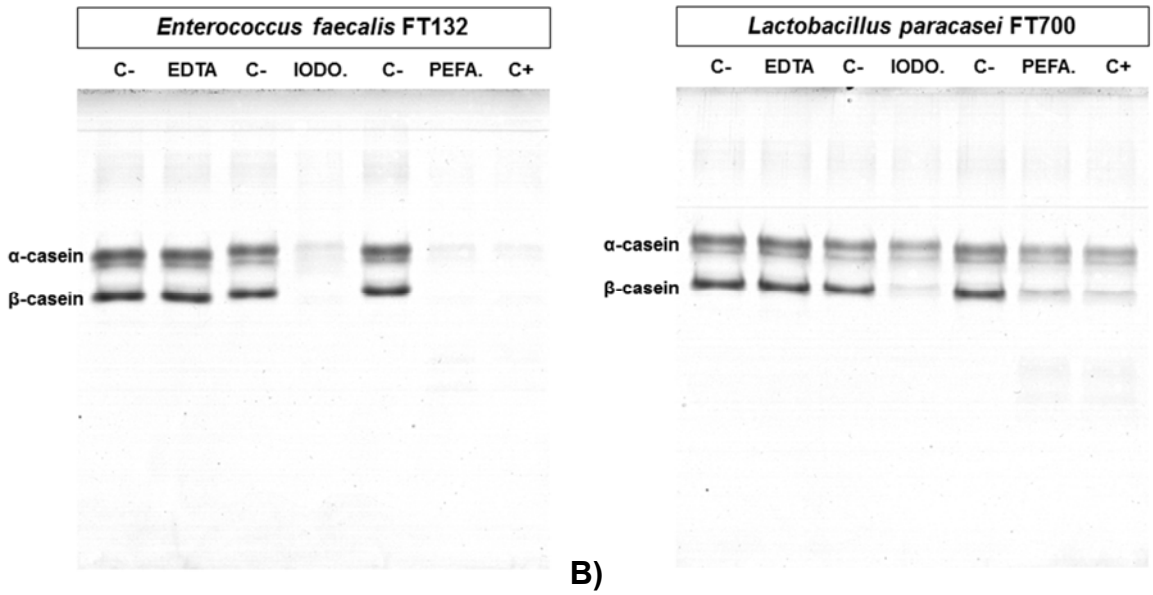


Figure 4.2 Photographies of proteolytic activities of *Enterococcus faecalis* FT132 (A) and *Lactobacillus paracasei* FT700 (B) on Na-caseinate after exposition to protease inhibitors, analyzed by SDS-PAGE. The reduction on the intensity of the bands in comparison to the negative control indicates that the LAB protease was not inhibited by the protease inhibitor tested. *C-: negative control (non-hydrolyzed Na-caseinate); C+: positive control (Na-caseinate hydrolyzed by the LAB); EDTA: EDTA was added to LAB suspension before proteolysis of Na-caseinate; IODO.: iodoacetic acid was added to LAB suspension before proteolysis of Na-caseinate; PEFA.: Pefabloc® was added to LAB suspension before proteolysis of Na-caseinate.

Table 4.3 Proteolytic activities of *Enterococcus faecalis* FT132 and *Lactobacillus paracasei* FT700 toward Na-caseinate or whey at different pH (37 °C, 24 h). The substrates were incubated with LAB in each condition and analyzed by SDS-PAGE. Results represent the comparison of visual intensity of the bands in polyacrylamide gels with non-hydrolyzed substrates

	pH 6.0		**pH 6.5**		**pH 7.0**	
	Na-caseinate	Whey	Na-caseinate	Whey	Na-caseinate	Whey
FT132	+++	+	+++	++	++	++
FT700	+++	+	+++	++	++	+

+ : Low proteolytic activity; + + : Medium proteolytic activity; + + + : High proteolytic activity

Table 4.4 Proteolytic activities of *Enterococcus faecalis* FT132 and *Lactobacillus paracasei* FT700 toward Na-caseinate or whey at different temperatures (pH 6.5, 24 h). The substrates were incubated with LAB in each condition and analyzed by SDS-PAGE. Results represent the comparison of visual intensity of the bands in polyacrylamide gels with non-hydrolyzed substrates

	30 °C		**37 °C**		**42 °C**	
	Na-caseinate	Whey	Na-caseinate	Whey	Na-caseinate	Whey
FT132	++	++	+++	++	+++	++
FT700	++	++	+++	++	+++	++

+ : Low proteolytic activity; + + : Medium proteolytic activity; + + + : High proteolytic activity

Table 4.5 Proteolytic activities of *Enterococcus faecalis* FT132 and *Lactobacillus paracasei* FT700 toward Na-caseinate or whey in the presence of different protease inhibitors (EDTA, iodoacetic acid and Pefabloc®) at 37 °C and pH 6.5. The substrates were incubated with LAB (previously exposed to protease inhibitors for 90 min) and analyzed by SDS-PAGE. Results represent the comparison of visual intensity of the bands in polyacrylamide gels with non-hydrolyzed substrates

	EDTA		**Iodoacetic acid**		**Pefabloc®**	
	Na-caseinate	Whey	Na-caseinate	Whey	Na-caseinate	Whey
FT132	inhibited	inhibited	not inhibited	not inhibited	not inhibited	not inhibited
FT700	inhibited	inhibited	not inhibited	not inhibited	not inhibited	not inhibited

The characterization of bacterial proteases is important for further applications in food industry, to evaluate how they are affected by food matrix, and how they act on food components. Williams and Banks (1997) evaluated the proteolytic activity of some NSLAB and found metalloproteases in *L. casei* strains, in addition to serine- and cysteine-proteases. In 2010, El-Ghaish et al. detected metalloproteases in *Enterococcus* strains, with proteolytic activity at pH 6.5 and 42 °C. In another study, Tsakalidou et al. (1999) evaluated the cell-wall bound proteinases of *Lactobacillus delbrueckii* subsp. *lactis* ACA-DC 178 by means of a cell-wall extract. Those authors detected the highest activity of crude proteinase extract was at pH 6.0 and 40 °C and characterized the bacterial enzyme as a serine-type proteinase.

In the present study, the proteolytic activity in each condition was similar for both strains (*E. faecalis* FT132 and *L. paracasei* FT700) and with both substrates. LAB hydrolyzed Na-caseinate and whey at pH 6.5, in the range of 37 to 42 °C. The best combination for proteolytic activity was pH 6.5 at 37 °C. In addition, the inhibition of proteolytic activity by EDTA, which is a metal chelator, suggests that both strains have metalloproteases that are responsible for Na-caseinate and whey hydrolysis. These findings are in accordance with the results from the literature, as previously presented.

3.2 HPLC analysis of hydrolyzed proteins

By HPLC analysis, it was possible to evaluate the proteolytic activity of selected LAB. The proteolysis would cause an increase in less hydrophobic peptides, in comparison to the non-hydrolyzed control. Na-caseinate and whey solutions hydrolyzed by *E. faecalis* FT132 or *L. paracasei* FT700 were analyzed by HPLC and the chromatograms are presented in Figures 4.3 and 4.4.

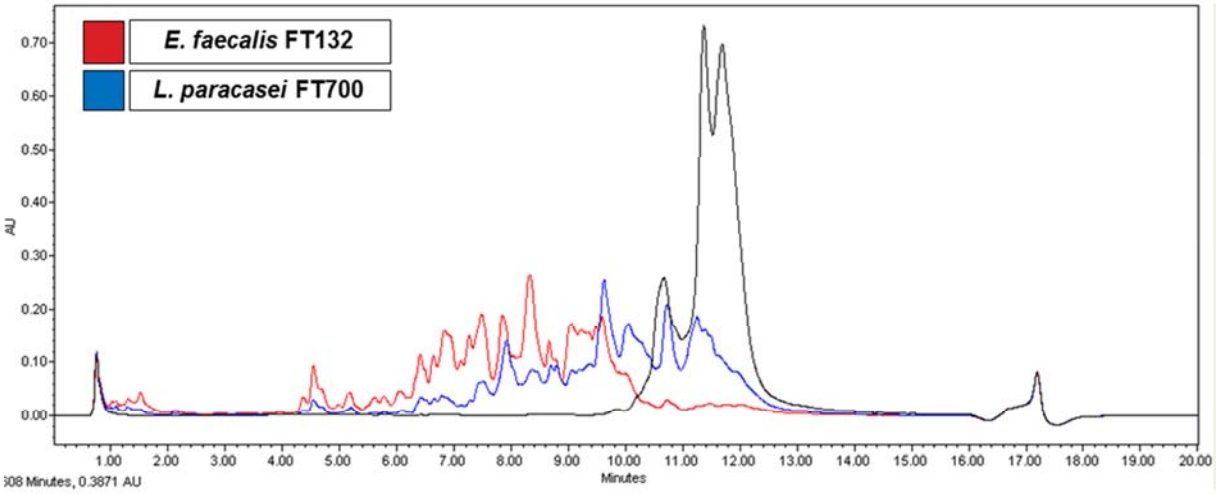


Figure 4.3 HPLC elution profile of Na-caseinate hydrolyzed by *Enterococcus faecalis* FT132 (red line) or *Lactobacillus paracasei* FT700 (blue line). Non-hydrolyzed Na-caseinate solution was used as negative control (black line). HPLC analysis was performed as described in item 2.2.5.

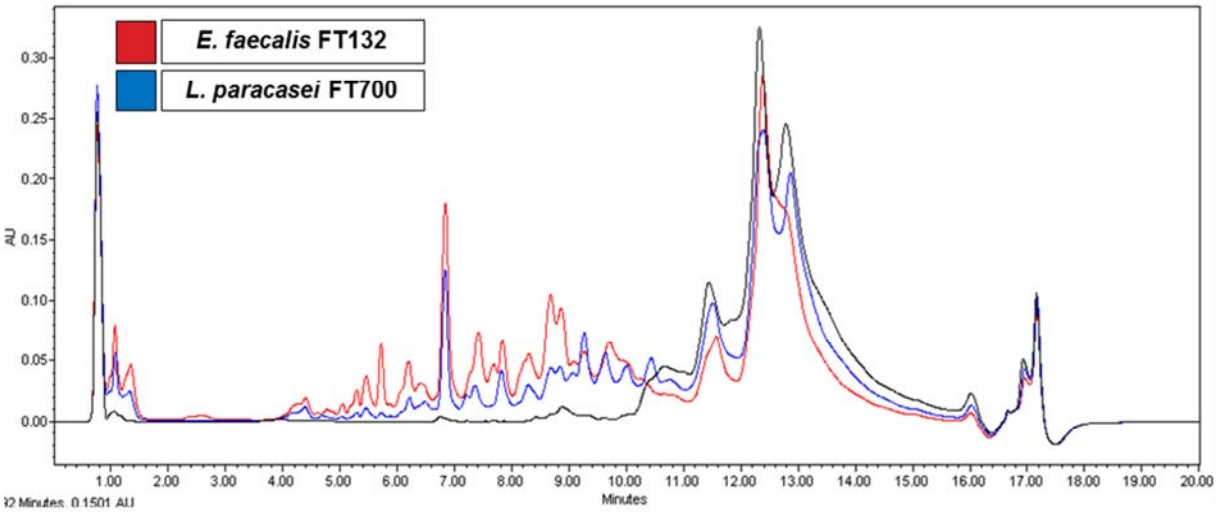


Figure 4.4 HPLC elution profile of whey hydrolyzed by *Enterococcus faecalis* FT132 (red line) or *Lactobacillus paracasei* FT700 (blue line). Non-hydrolyzed whey solution was used as negative control (black line). HPLC analysis was performed as described in item 2.2.5.

As presented in Figures 4.3 and 4.4, different patterns of proteolysis were obtained for Na-caseinate when comparing both strains, suggesting the presence of different proteolytic systems. El-Ghaish et al. (2010) obtained a complete hydrolysis of Na-caseinate using *E. faecalis* HH22, similar to the pattern observed for *E. faecalis* FT132 and presented in Figure 4.3. Similar data were also obtained by Sarantinopoulos, Kalantzopoulus and Tsakalidou (2002) when evaluating the use of *E. faecium* strains as adjunct cultures during Greek Feta cheese manufacture. After

HPLC analysis of water-soluble nitrogen extracts of fully-ripened Feta cheese (60 days), those authors observed an increase in the quantity of hydrophilic compounds and a decrease in the number of hydrophobic compounds, in comparison with the control without adjunct strains. Overall, the use of enterococci strains positively affected the taste, aroma, color and structure of fully ripened Feta cheeses (SARANTINOPOULOS; KALANTZOPOULUS; TSAKALIDOU, 2002). In this context, *E. faecalis* FT132 and *L. paracasei* FT700 may have potential to be used as adjunct cultures in cheese manufacture. However, virulence and pathogenic traits of *E. faecalis* FT132 should be extensively studied before application in food production. Some of these traits were evaluated and the results are presented as follows.

3.3 Virulence genes and antibiotic resistance in *E. faecalis* FT132

The virulence genes *asa*1, *ace* and *gel*E were detected in *E. faecalis* FT132 (Figure 4.5 and 4.6). According to Perin et al. (2014), the genes *asa*1 and *ace* are responsible for the production of substances that are involved in microbial colonization and adhesion to biotic and abiotic surfaces, and they are also involved in the evasion of the host immune system. Those authors observed high frequencies of *ace* and *asa*1 genes in enterococci and lactococci isolated from goat milk. Likewise, Gomes et al. (2008) evaluated 25 *E. faecalis* strains isolated from milk and detected high frequencies of the genes *ace* (100 %), *as* (84 %), *efa*A (100 %), *cyl*A (88.0 %) and *gel*E (96.0 %). The gene *gel*E is responsible for the production of an extracellular zinc metalloprotease that contributes for *E. faecalis* virulence in some animal models, and it is involved in hydrolysis of gelatin, collage and casein, among other proteinaceous substrates. Indeed, it is common to find this enzyme in enterococci isolated from milk and cheese, which is probably essential for these microorganisms to hydrolyze caseins (Lopes et al., 2006).

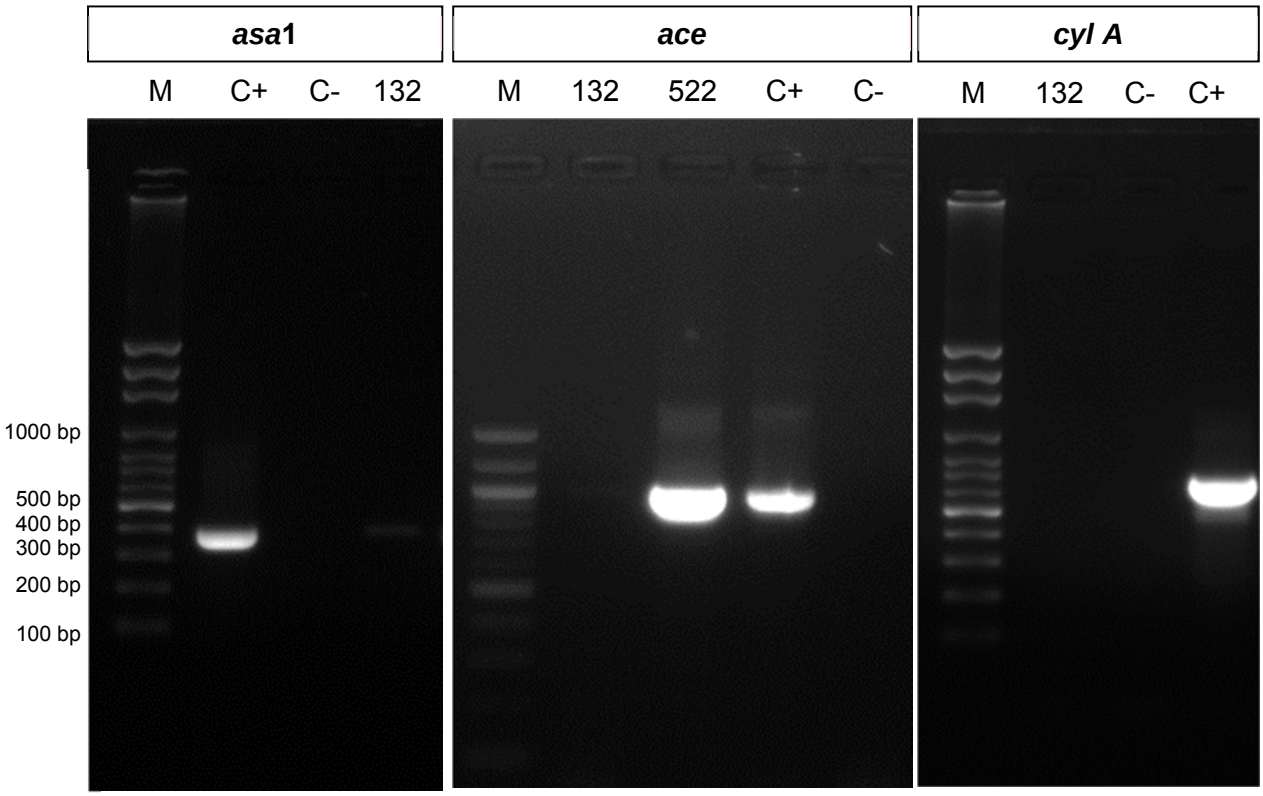


Figure 4.5 Photography of 1 % agarose gels showing the amplification products of the genes *asa*1, *ace*, and *cyl*A, using primers described in Table 4.2. In each lane, molecular ladder (M), positive control (C+), negative control (C-), *Enterococcus faecalis* FT132 ("132") and *Enterococcus faecalis* FT522 ("522").

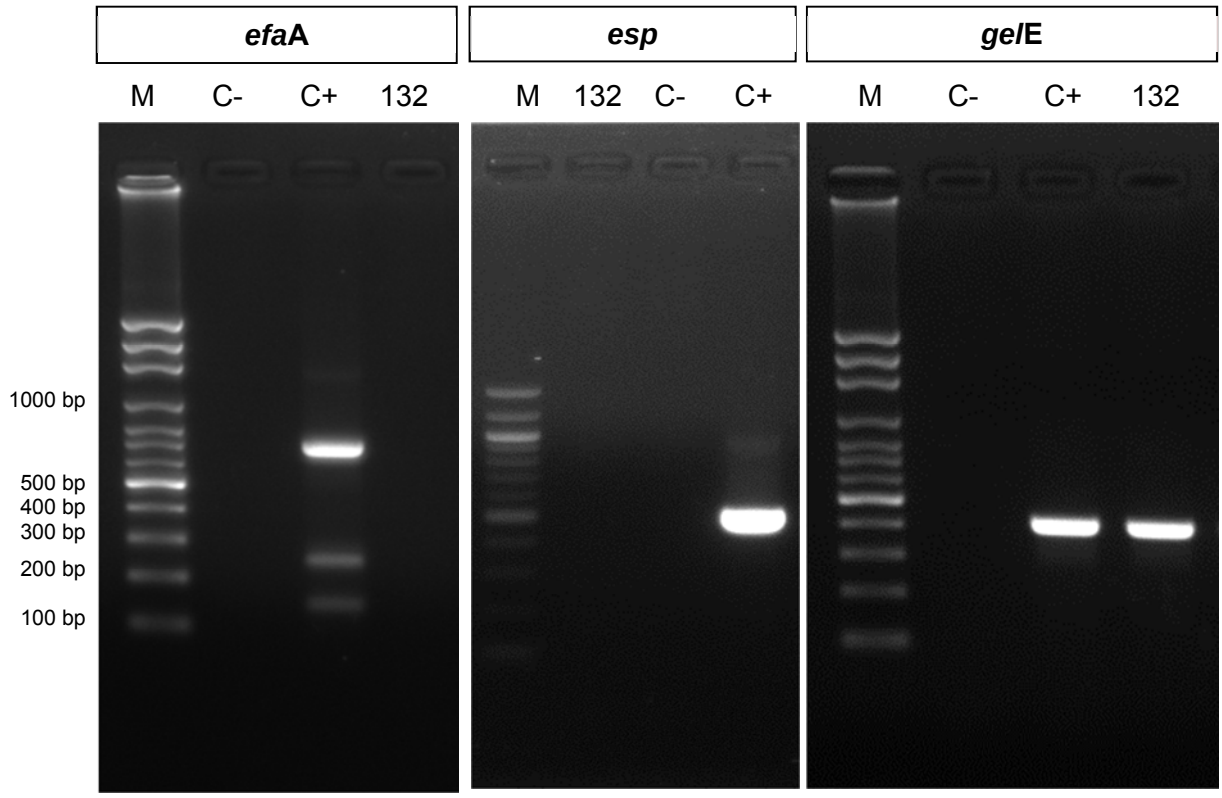


Figure 4.6 Photography of 1 % agarose gels showing the amplification products of the genes *efa*A, *esp* and *gel*E, using primers described in Table 4.2. In each lane, molecular ladder (M), positive control (C+), negative control (C-) and *Enterococcus faecalis* FT132 ("132").

With regard to antibiotic resistance, *E. faecalis* FT132 was sensitive to ampicillin, ciprofloxacin, chloramphenicol, penicillin, rifampicin and vancomycin, but it was resistant to erythromycin and tetracycline. In 2008, Gomes et al. evaluated the occurrence of antibiotic resistance among 80 *E. faecalis* isolated from Brazilian food. Those authors found that 31 %, 10 % and 22.5 % of the isolates were resistant to, respectively, tetracycline, erythromycin and gentamicin. Similarly, resistance to tetracycline and erythromycin was detected in *E. faecalis* FT132, while resistance to gentamicin was not evaluated. According to the Clinical and Laboratory Standards Institute (CLSI, 2012), aminoglycosides such as gentamicin may be scored active *in vitro*, but *Enterococcus* spp. can develop resistance to these antibiotics *in vivo*. Chloramphenicol, tetracycline and erythromycin resistances are a major concern for dairy *E. faecalis* isolates, despite only few ones are resistant to the clinically important antibiotics such as ampicillin, penicillin, gentamycin and vancomycin (OGIER; SERROR, 2008; TEUBER et al., 1999). Also, according to Ogier and Serror (2008), the presence of virulence genes does not mean that they are functional, but

the evaluation of antibiotic resistance and virulence factors may help to reduce undesirable enterococcal dissemination. Further studies on *E. faecalis* FT132 virulence genes and antibiotic resistance markers are necessary to guarantee the safe application of this microorganism or its metabolites in food products.

3.4 Antimicrobial activity of fermented milk supernatant (FMS)

Agar antagonism tests using the indicator strain *L. monocytogenes* IAL 633 revealed no antimicrobial activity of FMS for *E. faecalis* FT132 or *L. paracasei* FT700. However, Hayes et al. (2006) reported the existence of three antimicrobial peptides generated by the hydrolysis of Na-caseinate by a *Lactobacillus acidophilus* strain. The difference in antimicrobial activity observed in this study may be in part due to the panel of indicator microorganisms tested.

4. Conclusions

In this study, the proteolytic activities of two NSLAB (*L. paracasei* FT700 and *E. faecalis* FT132) isolated from dairy products were evaluated. The optimum pH and temperature were determined as well as the type of protease produced by both strains. Optimal conditions for proteolysis may contribute to obtain higher yields of hydrolyzed proteins when using these strains. The microbial safety of *E. faecalis* FT132 was also evaluated, and the strain was resistant to erythromycin and tetracycline, and harbored the virulence genes *asa*1, *ace* and *gel*E. The presence of such virulence traits is not appropriate for the application of *E. faecalis* FT132 in food. However, the peptides obtained by the proteolytic activity of this strain in milk may have some biological activity, and could be commercialized as purified formula. Thus, in the following chapter, studies on the biological activity on monocytes of peptides (fermented milk supernatant) produced by the proteolytic activity of *L. paracasei* FT700 and *E. faecalis* FT132 will be presented. In addition, the probiotic traits of *L. paracasei* FT700 were checked, as described in chapter 6. The use of *E. faecalis* FT132 as probiotic or adjunct culture in food products is not suitable due to the antibiotic resistance and the presence of virulence genes.

5. Influence of fermented milk supernatant produced by *Enterococcus faecalis* FT132 and *Lactobacillus paracasei* FT700 on monocytes: cytotoxicity, TNF-α production and differentiation into macrophages

Influence of fermented milk supernatant produced by *Enterococcus faecalis* FT132 and *Lactobacillus paracasei* FT700 on monocytes: cytotoxicity, TNF-α production and differentiation into macrophages

Abstract

Milk plays an important role in human nutrition and physiology, as its composition is sufficient to provide all essential amino acids necessary for protein metabolism. Bovine milk is composed of caseins and whey proteins and recent researches have focused on the biological activity of these proteins or their hydrolysates. One of the strategies to obtain dairy protein hydrolysates is to use the proteolytic activity of lactic acid bacteria (LAB), which have been used by mankind since immemorial times for the production of fermented dairy products due to their technological properties and ability to improve milk sensorial attributes. Thus, the aim of this study was to evaluate cytotoxicity and immunomodulatory effects on monocytes exposed to hydrolyzed milk proteins obtained after fermentation with two highly proteolytic strains, *Enterococcus faecalis* FT132 and *Lactobacillus paracasei* FT700, both isolated from Brazilian dairy products. Fermented milk supernatants produced by these strains were added to cell cultures (monocytes and macrophages) to evaluate their cytotoxicity, associated death mechanisms, immunomodulatory properties (macrophage differentiation) and TNF-α quantification. Fermented milk supernatants were toxic after 72 h of exposure at 10 mg/mL by apoptosis mechanism. Below cytotoxic concentrations, both cell-free supernatants of fermented milk produced by *E. faecalis* FT132 and *L. paracasei* FT700 stimulated the differentiation of monocytes into macrophages, as observed by the increased expression of CD71 marker. This immune stimulation was not inflammatory since low production of TNF-α was observed. Further studies are needed to evaluate these peptides in food matrices, and a study on the probiotic potential of *L. paracasei* FT700 will be presented in chapter 6.

Key-words: bioactive peptides, monocytes, *Enterococcus*, *Lactobacillus.*

Influência de sobrenadante de leite fermentado produzido por *Enterococcus faecalis* FT132 e *Lactobacillus paracasei* FT700 em monócitos: citotoxicidade, produção de TNF-α e diferenciação em macrófagos

Resumo

O leite possui um importante papel na nutrição e fisiologia humanas, visto que sua composição é suficiente para fornecer todos os aminoácidos essenciais necessários para o metabolismo de proteínas. O leite bovino é composto de caseínas e proteínas do lactosoro e, recentemente, várias pesquisas têm sido realizadas com foco na atividade biológica destas proteínas ou de seus hidrolisados. Uma das estratégias para a obtenção de hidrolisados de proteínas do leite é utilizar a atividade proteolítica das bactérias láticas. Esses micro-organismos têm sido utilizados pela humanidade há séculos para a produção de produtos lácteos fermentados devido às suas propriedades tecnológicas e capacidade para melhorar as propriedades sensoriais do leite. Desse modo, o objetivo deste estudo foi de avaliar a citotoxicidade e efeitos imunomoduladores sobre monócitos expostos a proteínas hidrolisadas do leite, obtidas pela fermentação do leite com duas linhagens bacterianas proteolíticas, *Enterococcus faecalis* FT132 e *Lactobacillus paracasei* FT700, ambas isoladas de produtos lácteos no Brasil. Para isso, os sobrenadantes de leite fermentado produzidos por estas linhagens foram adicionados às culturas de células (monócitos e macrófagos) para avaliar a sua citotoxicidade, o mecanismo de morte celular associado, propriedades imunomoduladoras (diferenciação de monócitos em macrófagos) e quantificação de TNF-α. Foi observado que essas proteínas hidrolisadas apresentam toxicidade após 72 h de exposição a 10 mg/mL e a morte celular ocorre por apoptose. Em concentrações menores que aquelas citotóxicas, ambos os sobrenadantes de leite fermentado produzidos por *E. faecalis* FT132 e *L. paracasei* FT700 estimularam a diferenciação de monócitos em macrófagos, como foi observado pelo aumento da expressão do marcador CD71. Esta estimulação não apresentou características de um processo inflamatório pois foi observada uma baixa produção de TNF-α. Outros estudos são necessários para avaliar estes peptídeos em matrizes alimentares, e o potencial probiótico de *L. paracasei* FT700 será apresentado no capítulo 6.

Palavras-chave: peptídeos bioativos, monócitos, *Enterococcus*, *Lactobacillus.*

Influence de surnageants de laits fermentés produits par *Enterococcus faecalis* FT132 et *Lactobacillus paracasei* FT700 sur des monocytes: cytotoxicité, production de TNF-α et différenciation en macrophages

Résumé

Le lait joue un rôle important en nutrition humaine, notamment car sa composition est suffisante pour fournir les acides aminés essentiels au métabolisme des protéines. Le lait de vache est composé de caséines et de protéines du lactosérum. Des recherches récentes ont mis l'accent sur les activités biologiques de ces protéines ou de leurs hydrolysats. Une des stratégies pour obtenir des hydrolysats de protéines laitières est d'utiliser l'activité protéolytique des bactéries lactiques. Celles-ci sont utilisées par l'humanité depuis l'antiquité pour la production de produits laitiers fermentés, en raison de leurs propriétés technologiques et de leur capacité à améliorer les propriétés organoleptiques du lait. Ainsi, l'objectif de cette étude était d'évaluer la cytotoxicité et les effets immunomodulateurs de deux hydrolysats de protéines laitières obtenus après fermentation de lait écrémé par deux souches de bactéries lactiques très protéolytiques, *Enterococcus faecalis* FT132 et *Lactobacillus paracasei* FT700. Ces souches ont été isolées à partir de produits laitiers Brésiliens. Les surnageants des laits fermentés par ces souches ont été ajoutés à des cultures cellulaires (monocytes et macrophages) pour évaluer leur cytotoxicité et les mécanismes de la mort cellulaire qui y sont associés, ainsi que leurs propriétés immunomodulatrices (différenciation des macrophages et quantification du TNF-α). Nous avons observé que les deux hydrolysats étaient cytotoxiques à forte concentration (10 mg/mL de surnageant lyophilisé) suite à 72 h d'exposition en favorisant l'apoptose des cellules. Au-dessous de ces concentrations, les deux surnageantes des laits fermentés produits par *E. faecalis* FT132 et *L. paracasei* FT700 ont stimulé la différenciation de monocytes en macrophages, comme observé par l'expression augmentée du marqueur CD71. Cette stimulation immunitaire n'était pas inflammatoire grâce à une faible production de TNF-α. Cependant, d'autres études seront nécessaires pour évaluer l'impact de ces peptides lors de leur production dans les matrices alimentaires, ainsi que le potentiel probiotique des souches productrices.

Mots-clés: peptides bioactifs, les monocytes, *Enterococcus*, *Lactobacillus.*

1. Introduction

Bovine milk is one of the most important protein source used to produce protein hydrolysates designed for clinical diets of patients with reduced absorptive capacity and food allergies (POTIER; TOMÉ, 2008). In addition, several studies have been done to evaluate the immunomodulatory effects of peptides derived from caseins and whey protein hydrolysis by digestive enzymes, via the modulation of both cell-mediated and humoral immunity (PARODI, 2007). Similarly, it was observed that during milk fermentation, bioactive peptides are produced from milk protein hydrolysis by LAB, and some of them also have immunomodulatory activities (DONKOR et al., 2007). Concerning industrial applications, these peptides may be considered as health enhancing nutraceuticals for food and pharmaceutical applications (KANWAR et al., 2009). In this context, the aim of this study was to evaluate the cytotoxicity and immunomodulatory effects on monocytes of hydrolyzed milk proteins obtained by the action of *Enterococcus faecalis* FT132 and *Lactobacillus paracasei* FT700, both isolated from Brazilian dairy products.

2. Materials and methods

2.1 Strains and culture conditions

The lactic acid bacteria (LAB) *Enterococcus faecalis* FT132 and *Lactobacillus paracasei* FT700 were previously isolated from Brazilian cow milk and goat cheese, respectively (chapter 3). They were screened for their proteolytic activity and identified by molecular tests (*16S rRNA* gene sequencing and polymerase chain reaction with species-specific primers), as previously presented in chapters 3 and 4). The strains were cultivated in MRS broth (De Man Rogosa Sharpe, AES Laboratory, France) at 37 °C for 24 h, and stored on MRS broth with 20 % v/v glycerol at -80 °C.

2.2 Production of fermented and acidified milk supernatants and analysis by high-performance liquid chromatography (HPLC)

For the production of fermented milk supernatant (FMS) containing hydrolyzed milk proteins, a 24 h culture of LAB incubated in MRS broth at 37 °C was washed

with NaCl 0.85 % w/v and inoculated into sterilized skim milk (Délisse, UHT skim milk, France) to obtain a final concentration of *ca.* 1 x 10^7 CFU/mL. The inoculated milk was incubated for 24 h at 37 °C and centrifuged at 8000 × *g* for 20 min at 4 °C. Fermented milk supernatants were collected, filtered through a 0.8 µm filter (Minisart, Sartorius Stedim Biotech, Germany), and desalted by using a C_8 cartridge (Sep-Pak Vac 20 cc 5 g, Waters, Ireland). The supernatants were loaded onto the cartridge previously conditioned with an aqueous solution containing 50 % v/v acetonitrile (Biosolve, Netherlands) and equilibrated with the solution A (aqueous solution with 0.03 % v/v trifluoroacetic acid, TFA, Sigma-Aldrich, USA). After a washing step with the solution A, hydrolyzed milk proteins were eluted with solution B (80 % v/v acetonitrile, 20 % v/v isopropyl alcohol, Sigma-Aldrich, and 0.03 % v/v TFA, JT Baker, USA) and freeze-dried. A non-fermented and non-hydrolyzed control (referred here as “acidified milk supernatant”, AMS) was produced with milk added of 4.3 g/L of lactic acid (Sigma-Aldrich) and purified as described above. In average, 4.3 g/L of lactic acid is produced in fermented milk by both strains, as measured according to instructions of manufacturer by the D/L Lactic Acid UV-Method Kit (Roche, Germany), and this concentration was used to produce the control AMS.

2.2.1 High-performance liquid chromatography (HPLC) analysis of fermented milk supernatant (FMS) and acidified milk supernatant (AMS)

Analyses by high-performance liquid chromatography (HPLC) of fermented milk supernatant (FMS) and acidified milk supernatant (AMS) were performed using a C_{18} column (5 µm, Symmetry 300 C_{18}, Waters, USA) in a Waters HPLC System Alliance. Chromatographic separation was conducted using water / acetonitrile / trifluoroacetic acid (95:5:0.05) as solution A, and isopropyl alcohol / acetonitrile / trifluoroacetic acid (20:80:0.03) as solution B. The flow rate was 1.2 mL/min and the peptides were detected at 220 nm in a Waters 996 photodiode array detector.

2.3 Mononuclear cells and culture conditions

Mononuclear cells originated from human umbilical cord blood samples were collected in citrated tubes from placentas after normal deliveries in Brest University Hospital MORVAN (France), with the consent of donors. Light density cells were

recovered by gradient density centrifugation (d = 1.077 g/mL) at 400 x *g* for 30 minutes, according to Hymery et al. (2009).

2.3.1 Monocytes

Monocyte culture conditions used to induce differentiation into macrophages were as follows. Monocytes were purified by a positive selection enrichment method (Miltenyi Biotech, Germany) using 5 x 10^7 mononuclear cells resuspended in 1 mL of phosphate buffered saline (PBS, Sigma) with 6 % v/v fetal bovine serum (FBS, Sigma). The cell suspension was incubated for 15 minutes at 4 °C with 100 µL Microbeads CD14 (Miltenyi Biotech). After labeling, monocytes were selected using positive selection columns (MS, Miltenyi Biotech) and cultured for 6 days in RPMI 1640 medium (Lonza, Germany) supplemented with 1 % L-glutamine, 1 % gentamicin, 1 % amphotericin (Gibco-BRL, Scotland) and 10 % inactivated FBS (Sigma). Before incubation in 24-well plates, 800 U/mL of granulocyte-macrophage colony-stimulating factor (GM-CSF, Miltenyi Biotech, specific activity: 1 x 10^7 U/mg) were added to the cell culture. Incubation was carried out in incubators at 37 °C in the presence of 5 % CO_2 and 100 % humidity.

2.3.2 Cytotoxicity assays (MTT)

The cytotoxic effect of FMS obtained with *E. faecalis* FT132 or *L. paracasei* FT700 was studied on monocytes, in comparison with the AMS. For that, monocytes were cultured in RPMI 1640 medium (Lonza) supplemented with 1 % L-glutamine, 1 % gentamicin, 1 % amphotericin (Gibco-BRL) and 10 % inactivated FBS (Sigma). FMS powder was resuspended in RPMI at 1, 5 and 10 mg/ml and incubated with monocytes during 24, 48 and 72 h at 37 °C, under 5 % CO_2 atmosphere and 100 % humidity. AMS was analyzed the same way as a non-hydrolyzed protein control.

Cytotoxicity toward monocytes primary culture was evaluated using the Promega CellTiter 96AQ_{ueous} One cell proliferation assay (Promega, USA), as described by Hymery et al. (2014), with slight modifications. This colorimetric method determines cell viability based on the reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) to formazan by mitochondrial dehydrogenases present only in viable cells.

After 24, 48 or 72 h of incubation in the presence of FMS or AMS, monocytes were washed with PBS, resuspended in 100 µL of the same buffer, and seeded in 96-well plates at 250,000 cells/well. Next, 20 µL of CellTiter 96AQ$_{ueous}$ One Solution were added to each well and the monocytes were further incubated for 3 h at 37 °C, under 5 % CO_2 atmosphere and 100 % humidity. Formazan was quantified by reading absorbance at 450 nm on a Multiskan FC plate reader (Thermo Scientific, USA). Each experiment was performed in biological triplicate and technical triplicate on cells from three different umbilical cord blood samples.

2.3.3 Mechanism of cytotoxicity: apoptosis study

The mechanism of monocyte death was evaluated by the Annexin V-FITC/PI kit (Miltenyi). Monocytes were cultured as described previously, in the presence of FMS. Then, cells were collected, washed with the binding buffer (provided by the kit) and stained with annexin-V FITC and propidium iodide (PI, Miltenyi). Cells were analyzed on a C6 Accuri BD Flow Cytometer using Accuri Software (Becton Dickinson, USA). FITC-fluorescence (FL1-H) was collected through a 530/30 nm band-pass filter and IP fluorescence (FL3-H) through a 660/20 nm band-pass filter. Since double labeling was applied to cells, the compensation between FL1 and FL3 was used to reduce artifacts due to the overlapping of channels.

2.3.4 Effect on macrophage differentiation

Monocytes were cultured for 6 days in RPMI 1640 medium (Lonza, Germany) supplemented with 1 % L-glutamine, 1 % gentamicin, 1 % amphotericin (Gibco-BRL) and 10 % inactivated FBS (Sigma) in the presence of 800 U/mL of GM-CSF (Miltenyi Biotech, specific activity: 1 x 10^7 U/mg). Incubation conditions corresponded to 37 °C, 5 % CO_2 and 100 % humidity. Effect on macrophage differentiation was evaluated with non-cytotoxic concentrations (previously evaluated by MTT assay, item 2.3.2) of FMS or AMS at 10 and 1 mg/mL. After the exposition, cells were analyzed by flow cytometry as follows.

2.3.4.1 Flow cytometric immunolabeling assays

Phenotypic expression was determined by flow cytometric immunolabeling assays. CD14 and CD71 expression in monocytes/macrophages were analyzed after 6 days of culture as described previously (item 2.3.4). Cells were incubated for 30 minutes at 4 °C in PBS supplemented with 6 % FBS and with fluorochrome-conjugated mouse monoclonal antibodies (Fluorescein isothiocyanate FITC-conjugated anti-CD14, R-phycoerythrin R-PE-conjugated anti-CD71) or with isotype-matched mouse control antibodies (Miltenyi Biotech). Cells were analyzed on a flow cytometer C6 Accuri (Becton Dickinson). FITC-fluorescence (FL1-H) was collected through a 530/30 nm band-pass filter and R-PE fluorescence (FL2-H) through a 660/20 nm band-pass filter.

2.3.4.2 TNF-α quantification

The production of TNF-α (Tumor necrosis factor) cells exposed to the FMS and AMS was evaluated by using the Human TNF-alpha enzyme-linked immunosorbent assay kit (Promocell, Germany). Figure 5.1 represents the sequence of analyses carried out during this study.

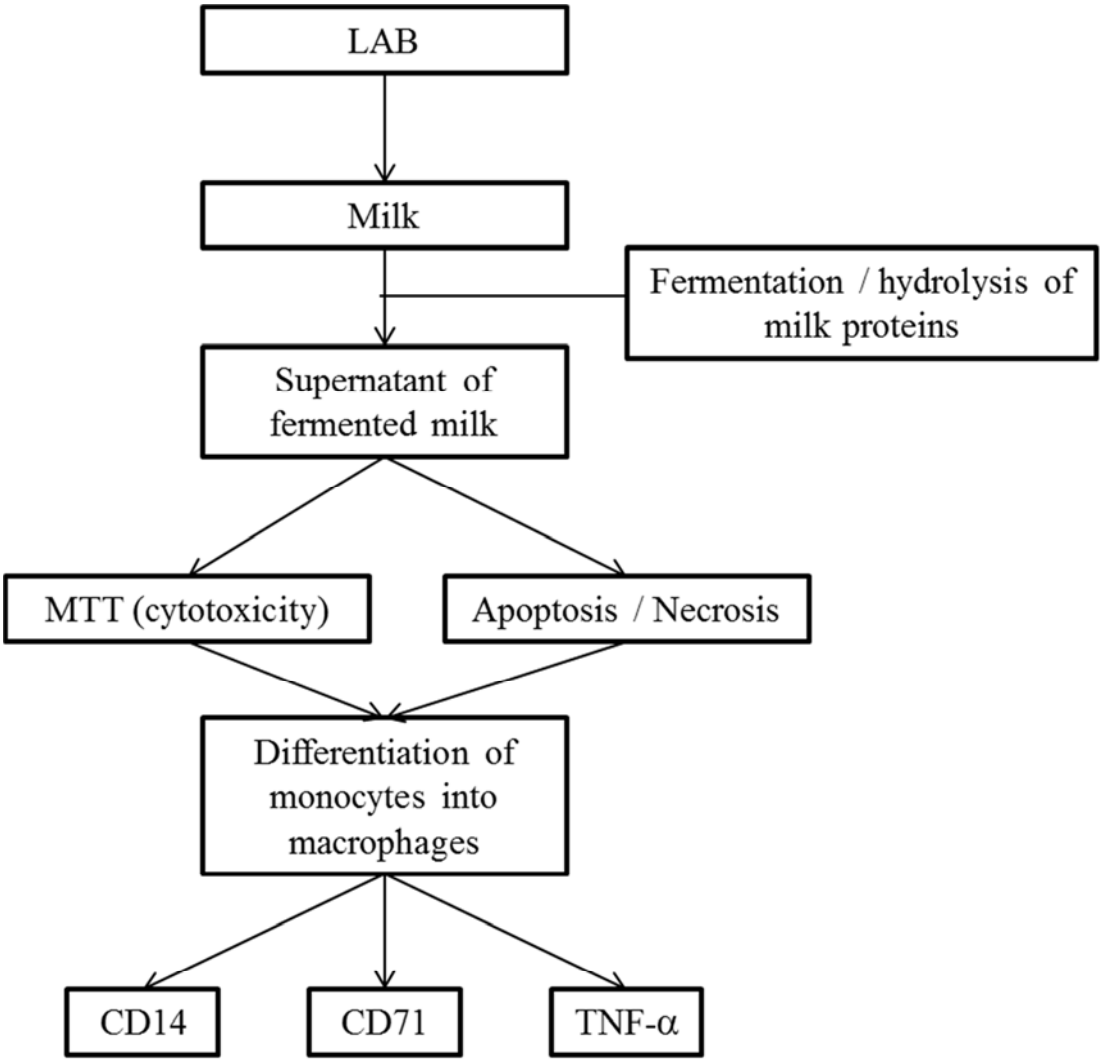


Figure 5.1 Flowchart of the analyses performed during this study.

2.3.5 Statistical analysis

Different mean value groups were compared to control values according to the least significant differences (LSDs) test of multifactor analysis of variance (ANOVA) using Statgraphic Plus for Windows (version 1.4 StatPoint Technologies Inc., Warrenton). Theoretical concentration-response curves were determined with the Microsoft Table-Curve Software and IC50 determination was carried out. Each experiment was performed in three independent biological replicates. Three technical replicates were done for each biological replicate. Standard deviations for each experiment were established from three biological replicates.

3. Results and discussion

3.1 HPLC analyses of fermented milk supernatant (FMS) and acidified milk supernatant (AMS)

The control with non-hydrolyzed proteins (AMS, produced by the addition of lactic acid to skim milk) and the fermented milk supernatant (FMS, produced by *E. faecalis* FT132 and *L. paracasei* FT700) were prepared, desalted by C_8 cartridge elution and analyzed by reverse phase HPLC (C_{18} column). The elution profiles of FMS and AMS are represented in Figures 5.2 and 5.3, and compared in the same chromatogram with the elution profiles of non-hydrolyzed Na-caseinate and whey proteins. The elution profiles of hydrolyzed proteins (FMS) presented considerable less hydrophobic peptides (compounds eluted at the beginning of the process) in comparison with non-hydrolyzed proteins (AMS). The majority of peptides eluted between 4 and 10 minutes, with reduction of peaks related to non-hydrolyzed Na-caseinate and whey proteins, between 10 and 14 minutes. In the elution profile of *E. faecalis* FT132 fermented milk supernatant, it was detected a group of major peaks around 6 minutes that were not so intense in the elution profile of *L. paracasei* FT700 fermented milk supernatant. However, two peaks between 10 and 11 minutes were detected for *L. paracasei* FT700, but not for *E. faecalis* FT132, indicating a different protein composition between both FMS. Overall, the chromatograms presented in Figures 5.2 and 5.3 confirm the hypothesis that *E. faecalis* FT132 has a proteolytic system more active than *L. paracasei* FT700. El-Ghaish et al. (2010) evaluated the proteolytic activity of *E. faecalis* HH22 and *Enterococcus faecium* DO623 on Na-caseinate by HPLC. Those authors also observed a large peak at the beginning of the chromatogram, while the peaks representing the native Na-caseinate had almost disappeared. Similarly, Pescuma et al. (2011) evaluated the proteolytic activity of *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 656 on β-lactoglobulin, with the majority of peptides of hydrophilic nature, indicated by early elution from C_{18} column. The results presented in this study for milk protein hydrolysis by LAB corroborate these findings from the literature.

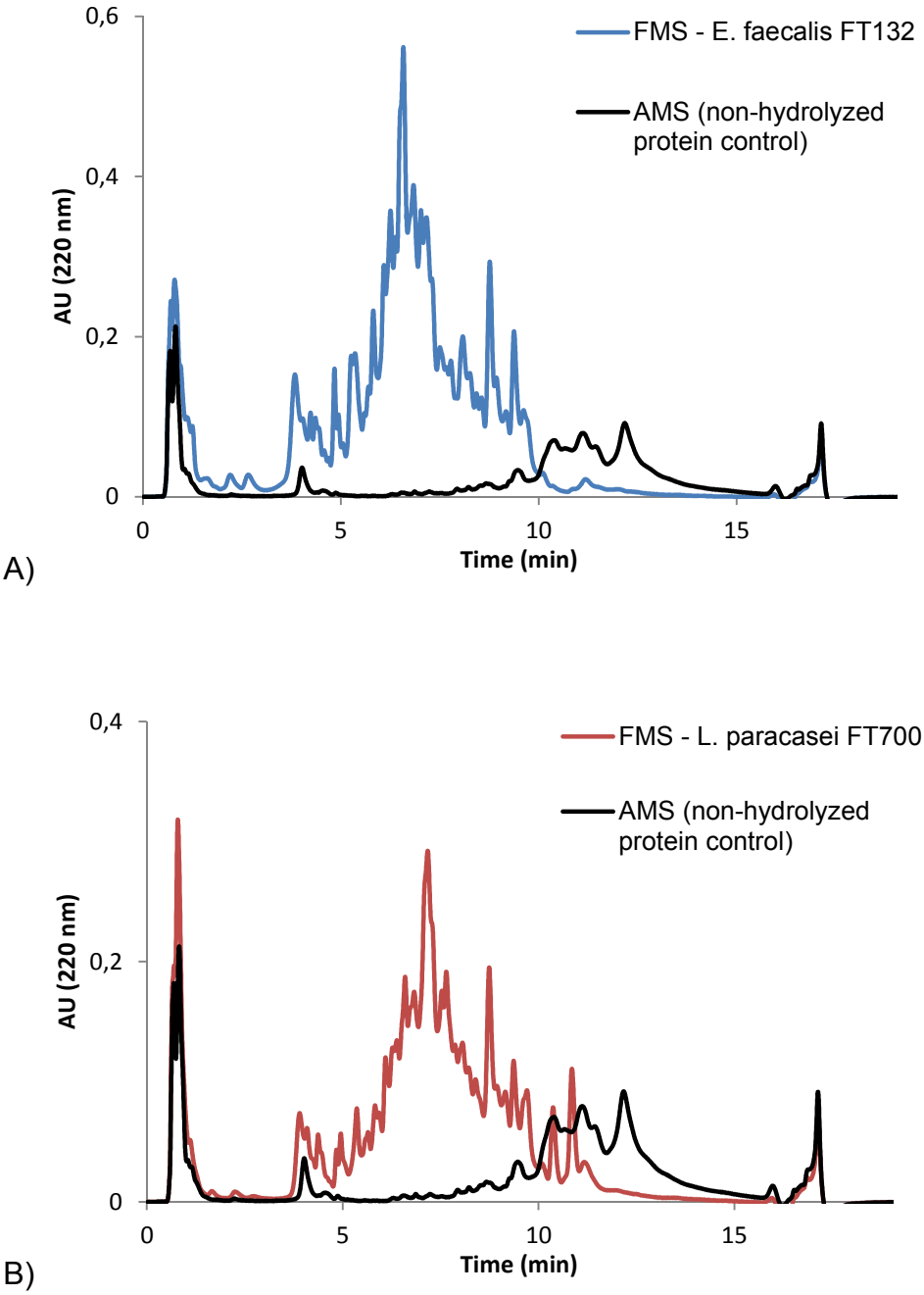


Figure 5.2 HPLC elution profiles of desalted fermented milk supernatant (FMS) produced by *Enterococcus faecalis* FT132 (A) and *Lactobacillus paracasei* FT700 (B), compared with acidified milk supernatant (AMS, non-hydrolyzed protein control).

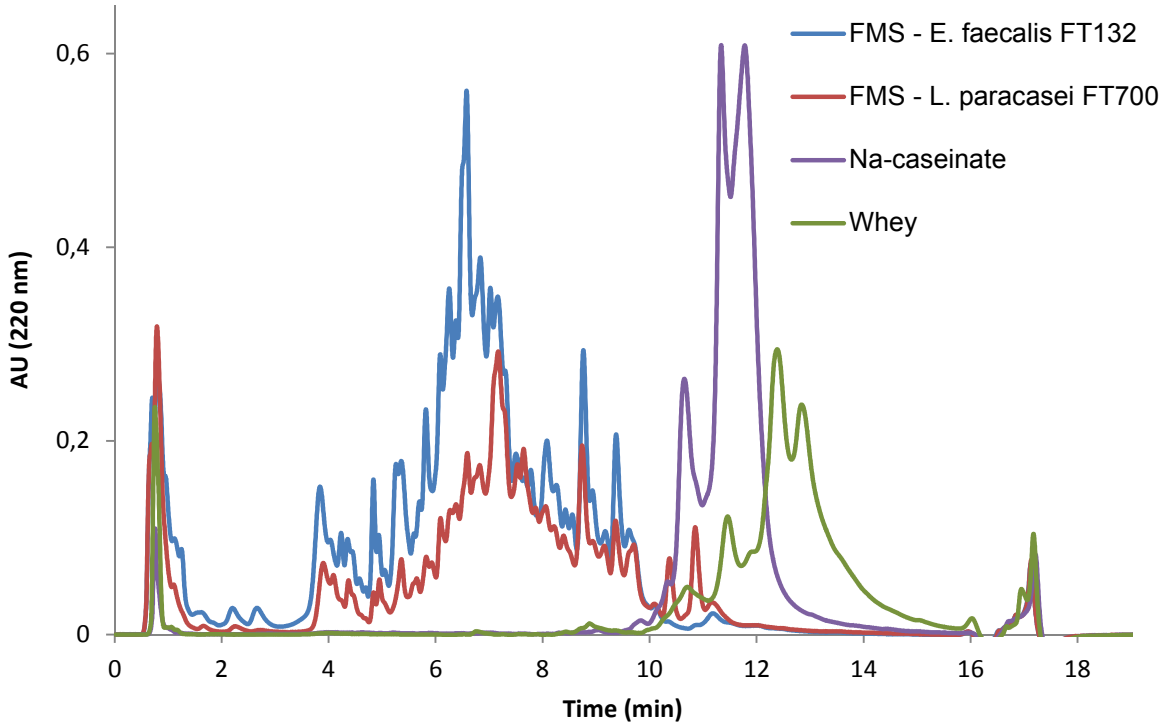


Figure 5.3 Comparison of HPLC elution profiles of desalted fermented milk supernatant (FMS) produced by *Enterococcus faecalis* FT132 (blue line) and *Lactobacillus paracasei* FT700 (red line), non-hydrolyzed Na-caseinate (purple line) and whey (green line).

3.2 Cytotoxicity assays and cell death mechanism

The initial step to evaluate the influence of FMS on monocytes was to determine the lowest toxic concentrations that would still have some effect on monocytes. Concentrations to be tested were chosen according to a similar study carried out by Cross and Gill (1999) that evaluated the cytotoxicity of a modified whey protein concentrate toward splenic murine leucocytes using up to 10 mg/mL for 48 h. In our study, concentrations of 1, 5 and 10 mg/mL were initially tested. The results obtained from cytotoxicity assays using FMS and AMS are presented in Table 5.1.

Table 5.1 Results for cytotoxicity tests of acidified milk supernatant (AMS) and fermented milk supernatants (FMS, obtained from *Enterococcus faecalis* FT132 and *Lactcobacillus paracasei* FT700) on monocytes, after 24, 48 and 72 h of exposition. Results are expressed in percentage of viable cells

	Enterococcus faecalis FT132					
	1 mg/mL		5 mg/mL		10 mg/mL	
	FMS	AMS	FMS	AMS	FMS	AMS
24 h	96.7 ± 14.9 [a]	102.4 ± 0.4	80.0 ± 17.9 [a]	80.9 ± 13.6	67.0 ± 18.3 [b, c]	84.3 ± 16.8
48 h	90.5 ± 17.9	84.7 ± 1.1	75.3 ± 26.1	70.0 ± 13.9	53.5 ± 19.1 [b, c]	75.0 ± 13.8
72 h	91.5 ± 16.0	60.3 ± 5.6 [b]	70.0 ± 17.2	53.9 ± 12.6 [b]	50.7 ± 13.2 [b,c]	59.6 ± 10.5 [b]

	Lactobacillus paracasei FT700					
	1 mg/mL		5 mg/mL		10 mg/mL	
	FMS	AMS	FMS	AMS	FMS	AMS
24 h	84.8 ± 10.6	103.0 ± 6.4	81.2 ± 17.9 [a]	75.9 ± 14.1	64.6 ± 11.4 [b]	83.4 ± 18.2
48 h	96.5 ± 16.7 [a]	67.4 ± 5.7 [b]	73.9 ± 21.5	68.5 ± 14.8	57.7 ± 14.9 [b, c]	76.1 ± 11.9
72 h	84.6 ± 10.9	50.1 ± 4.8 [b]	68.1 ± 18.8 [b]	57.8 ± 10.3 [b]	51.7 ± 12.7 [b, c]	60.4 ± 12.5 [b]

"a" statistically different from AMS at the same concentration; "b" different from 100 % of viability; "c" different from 1 mg/mL. Cytotoxicity was expressed as compared to control (only RPMI in cell culture) representing 100% of viability. ANOVA was calculated per exposure time (P<0.05).

The exposition of monocytes to FMS-FT132 at 1 and 5 mg/mL induced similar modification in cell viability. The FMS-FT132 at 10 mg/mL in all exposition times was more toxic than the FMS-FT132 at 1 mg/mL and the AMS at 10 mg/mL, as observed by the low cell viability of 50.7 ± 13.2 %. Cytotoxic effects of FMS-FT700 were similar to those obtained with FMS-FT132, presenting less toxicity at 1 mg /mL and more toxic effects at 10 mg/mL. Taken together, the results for FMS (72 h of exposition) showed cell viability was reduced to about 50 % at 10mg/mL, while no toxic effect was observed for FMS at 1 mg/mL. The AMS was also toxic to monocytes at all concentrations tested (72h of exposition). The concentrations of FMS at 1 and 10 mg/mL were chosen for the next analysis on differentiation of monocytes into macrophages.

To evaluate cell death mechanism, the cells exposed to hydrolyzed milk proteins were stained with annexin V and propidium iodide. In early apoptotic cells, phosphatidylserine turns from the inner surface to the external surface of plasma membrane, and then annexin V binds with high affinity to this phospholipid (BEDNER

et al., 1999). On the other hand, propidium iodide binds stoichiometrically to nucleic acids (RICCARDI; NICOLETTI, 2006) and thus, cells with damaged plasma membrane, late apoptotic and necrotic cells are stained with propidium iodide. According to Bedner et al. (1999), apoptotic cells are labeled only with annexin, and necrotic cells are stained with both dyes. The FMS-FT132 at the concentration of 1 and 10 mg/mL did not increase apoptosis in monocytes. However, a higher level of apoptosis was observed in monocytes exposed to FMS-FT700 at 1 mg/mL. With regard to necrotic cells, both hydrolyzed proteins increased the level of necrosis in monocytes when used at 1 and 10 mg/mL, except for the FMS-FT132 at 1 mg/mL. The percentage of apoptotic and necrotic cells in cultures exposed to AMS were not statistically different from cultures that were not exposed to milk proteins (0 mg/mL), as presented in Tables 5.2 and 5.3. Figure 5.4 illustrates the results for the analysis of a single flow cytometry experiment.

Table 5.2 Apoptosis on monocytes exposed to AMS and FMS (obtained from *Enterococcus faecalis* FT132 and *Lactobacillus casei* FT700), at 1 and 10 mg/mL after 120 h. Results are expressed in percentage of labelled apoptotic cells *

	1 mg/mL	10 mg/mL
FMS-FT132	12.9 ± 3.3	12.9 ± 3.7
FMS-FT700	20.1 ± 1.3[a]	15.6 ± 5.6
AMS	11.5 ± 3.5	10.8 ± 4.0

"a" statistically different from 0 mg/mL (only RPMI in cell culture). Biological triplicate statistics in a row (P<0.05). *Statistical analysis was done in comparison to 12.2 ± 2.3 % apoptotic cells detected among monocytes not exposed to milk proteins (only RPMI in cell culture).

Table 5.3 Necrosis on monocytes exposed to AMS and FMS (obtained from *Enterococcus faecalis* FT132 and *Lactobacillus casei* FT700), at 1 and 10 mg/mL after 120 h. Results are expressed in percentage of labelled necrotic cells *

	1 mg/mL	10 mg/mL
FMS-FT132	4.0 ± 1.3	8.1 ± 1.2[a,b]
FMS-FT700	4.6 ± 0.8[a]	9.8 ± 1.3[a,b]
AMS	2.4 ± 1.5	1.2 ± 2.0

"a" statistically different from 0 mg/mL (only RPMI in cell culture); "b" statistically different from 1 mg/mL. Biological triplicate statistics in a row (P<0.05). *Statistical analysis was done in comparison to 2.1 ± 0.2 % necrotic cells detected among monocytes not exposed to milk proteins (only RPMI in cell culture).

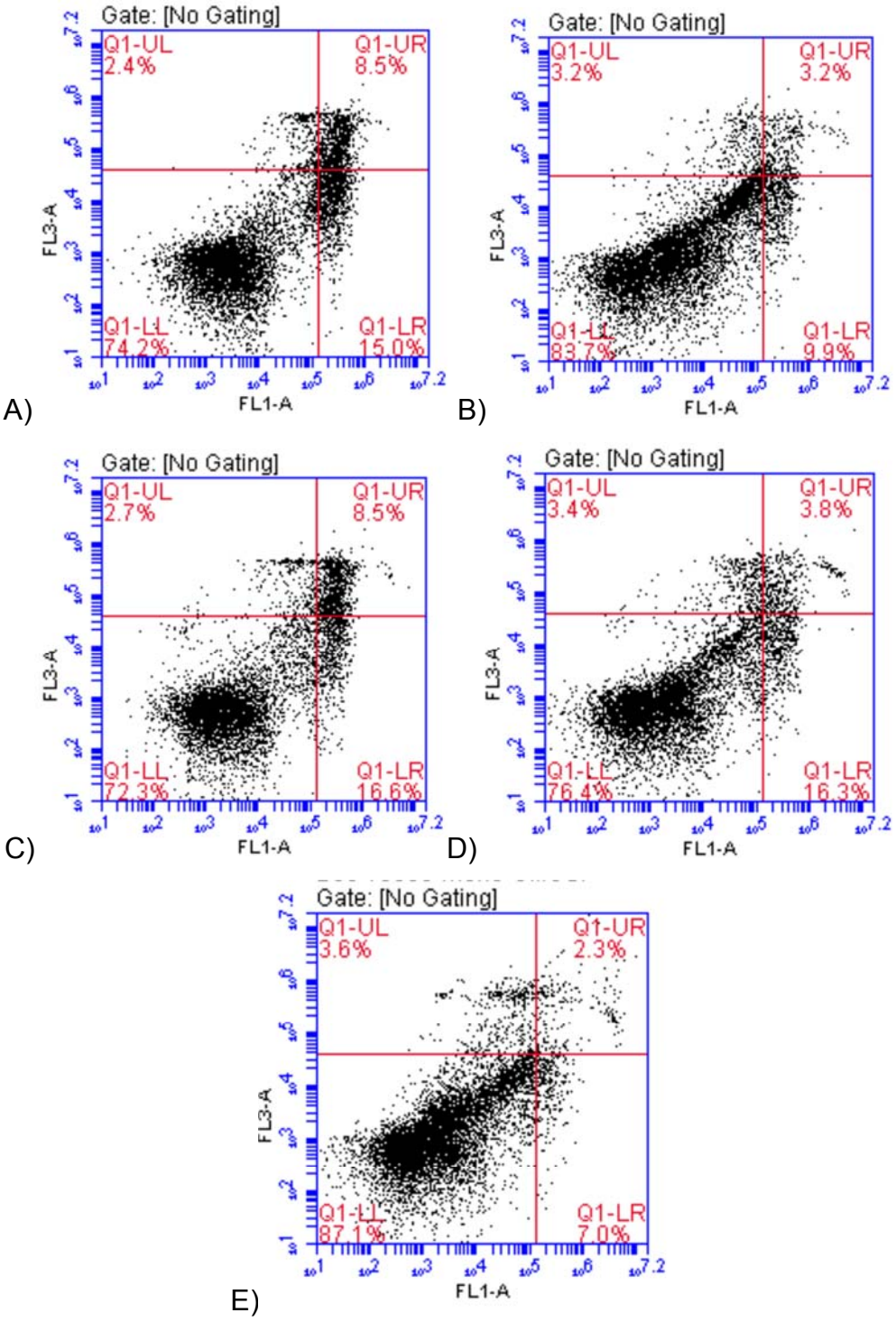


Figure 5.4 Diagrams from flow cytometry analyses of cells exposed to (A) 1 mg/mL of FMS-FT132 and (B) 10 mg/mL of FMS-FT132; (C) 1 mg/mL of FMS-FT700 and (D) 10 mg/mL of FMS-FT700; (E) control (no exposure to milk proteins). Cells were stained with annexin V (axis FL1-A) / propidium iodide (axis FL3-A) to evaluate the mechanism of cell death. The upper right square represents necrotic cells (stained with annexin and propidium iodide), and the lower right square represents apoptotic cells (stained only with annexin). Viable cells are represented in the lower left square.

3.3 Effect on monocyte differentiation into macrophages and TNF-α quantification

CD71 is a cell marker expressed by macrophages but not by monocytes, while CD14 is a monocyte cell marker, which may also be expressed in macrophages. An increased expression of CD71 indicates that monocytes are stimulated to differentiate into macrophages (HYMERY et al., 2009). In this study, for both strains, the FMS at 1 mg/mL increased the expression of CD71 when compared to AMS and cells not exposed to milk proteins. However, both FMS and AMS at 10 mg/mL had no effect on CD71 expression when compared to the control without milk proteins (Tables 5.4 and 5.5). Acidified milk supernatant greatly decreased the expression of CD14 in cells exposed to 1 mg/mL of AMS, which could indicate an inhibition on the differentiation of monocytes into macrophages. This process, however, was not observed in cells exposed to FMS produced by both strains.

Table 5.4 Expression of CD71 cell marker on monocytes exposed to FMS obtained from *Enterococcus faecalis* FT132 and *Lactobacillus paracasei* FT700, and the non-hydrolyzed protein control AMS, at 1 and 10 mg/mL, after 120 h of exposition. Results are expressed in arbitrary units (AU) of fluorescence *

	CD71	
	1 mg/mL	**10 mg/mL**
FMS-FT132	20575.2 ± 2855.6 [a,b]	9544.2 ± 988.3 [c]
FMS-FT700	16191.5 ± 796.5 [a,b]	8765.4 ± 736.2 [c]
AMS	7434.4 ± 767.0	8005.7 ± 2629.6

"a" statistically different from AMS; "b" statistically different from 0 mg/mL (only RPMI in cell culture); "c" statistically different from 1 mg/mL; $P<0.05$. *Expression of CD71 for control (monocytes not exposed to milk proteins, *i.e.* 0 mg/mL) was 8697.3 ± 2737.0 AU.

Table 5.5 Expression of CD14 cell marker on monocytes exposed to FMS obtained from *Enterococcus faecalis* FT132 and *Lactobacillus paracasei* FT700 and the non-hydrolyzed protein control AMS, at 1 and 10 mg/mL, after 120 h of exposition. Results are expressed in arbitrary units (AU) of fluorescence *

	CD14	
	1 mg/mL	**10 mg/mL**
FMS-FT132	21204.1 ± 3611.3 [a]	20564.8 ± 3535.7
FMS-FT700	15948.8 ± 2879.5	15625.6 ± 1399.7
AMS	10972.3 ± 1629.0 [b]	14341.7 ± 5285.3

"a" statistically different from AMS; "b" statistically different from 0 mg/mL (only RPMI in cell culture); "c" statistically different from 1 mg/mL; $P<0.05$. *Expression of CD14 for controls (monocytes not exposed to milk proteins, *i.e.* 0 mg/mL) was 20993.8 ± 4157.6 AU.

TNF-α is a potent proinflammatory cytokine with broad-ranging functions, regulating endothelial cell adhesion molecules to facilitate the access of leucocytes into tissues and inducing cellular cytotoxicity (KÖRNER; SEDGWICK, 1996). Also, there is a small production of TNF-α when monocytes differentiate into macrophages, but when an inflammatory process occurs, higher levels of this cytokine may be detected (HYMERY et al., 2009). The production of TNF-α by monocytes exposed to the FMS was quantified to evaluate their response to the hydrolyzed proteins. The production of TNF-α by monocytes stimulated with GM-CSF was not affected by the presence of FMS and AMS, compared to monocytes with GM-CSF only, Table 5.6, which indicates milk proteins would not cause inflammation. Moreover, since stimulation of monocyte differentiation was indicated by the increased expression of CD71 (Table 5.4), these data combined suggest milk fermented by *L. paracasei* FT700 or *E. faecalis* FT132 (FMS) could be consumed to enhance gut immunity. This encourages *in vivo* tests to be conducted to confirm these properties.

Table 5.6 TNF-α production by monocytes exposed to FMS obtained from *Enterococcus faecalis* FT132 and *Lactobacillus paracasei* FT700 and the non-hydrolyzed protein control AMS, after 120 h of exposition, expressed in pg/mL *

	1 mg/mL	**10 mg/mL**
FMS-FT132	169 ± 19	156 ± 8 [a]
FMS-FT700	206 ± 26	121 ± 10 [a]
AMS	143 ± 13 [a]	176 ± 77

"a" statistically different from 0 mg / mL (only RPMI in cell culture); $P<0.05$. *Monocytes not exposed to milk proteins (*i.e.* 0 mg/mL) produced 243 ± 56 pg/mL of TNF-α.

With regard to increased expression of CD71 in monocytes exposed to FMS, the results of the present study are similar to other found in literature employing milk proteins hydrolyzed by commercial enzymes or LAB. In a recent paper, Vordenbäumen et al. (2013) evaluated the role of human casein alpha S1 (CSN1S1) on the differentiation of monocytes toward macrophages. Those authors observed that the casein fraction increased the adherence capacity of cells, phagocytic activity, and also increased the differentiation of monocytes either into macrophages or into dendritic cells. Similarly, Kitazawa et al. (2007) demonstrated that enzymatic digestion of β-casein released potent chemotactic peptides that promote innate host immune response by activation and migration of macrophages. Matar et al. (2001) compared the immunomodulatory effects of milk fermented with a *Lactobacillus helveticus* strain and its non-proteolytic variant. Those authors reported that milk fermented with the proteolytic strain was able to enhance the immune system of mice that received the product orally, but no effect was observed for animals fed with milk fermented with the non-proteolytic LAB variant.

4. Conclusions

The proteolytic activity of *E. faecalis* FT132 and *L. paracasei* FT700 on milk proteins was confirmed by HPLC analyses, which indicated the FMS presented smaller peptides in comparison with AMS. When tested in monocyte cultures, the FMS from both strains at lower concentrations (1 mg/mL) stimulated the differentiation of monocytes into macrophages, as observed by the increased expression of CD71 cell marker. This stimulation was not inflammatory, since production of TNF-α was maintained low. These results indicate that immune response may be stimulated by these hydrolyzed proteins, resulting from fermentation. *E. faecalis* FT132 has a dualistic role in food microbiology and it may not be suitable for use as probiotic or adjunct culture in dairy products due to safety concerns, but the hydrolyzed milk proteins might be an ingredient for purified formulas. On the other hand, *L. paracasei* FT700 has a GRAS (generally recognized as safe) status and was tested for its probiotic potential, as demonstrated in the next chapter.

6. Preliminary evaluation of the probiotic potential of *Lactobacillus paraplantarum* FT259 and *Lactobacillus paracasei* FT700, isolated from Brazilian cheeses

Preliminary evaluation of the probiotic potential of *Lactobacillus paraplantarum* FT259 and *Lactobacillus paracasei* FT700, isolated from Brazilian cheeses

Abstract

Two lactobacilli isolated from Brazilian dairy products (*Lactobacillus paraplantarum* FT259 and *Lactobacillus paracasei* FT700, chapter 3) were tested for their potential application as probiotics in fermented dairy products. Previous studies showed that *L. paracasei* FT700 produced peptides with immunomodulatory properties from the proteolysis of milk proteins (chapter 5), and *L. paraplantarum* FT259 produced antilisterial bacteriocins (chapter 3). The strains were tested for survival in acidified culture media (pH 2.0, 2.5 and 3.5), *in vitro* tolerance to bile salts, survival in simulated gastric juice and antibiotic susceptibility. In addition, the antimicrobial peptides produced by *L. paraplantarum* FT259 were partially purified in a column filled with XAD-16 resin, followed by solid phase extraction in C_{18} cartridge. The partially purified peptides were analyzed by sodium dodecil sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Polymerase chain reactions with primers for plantaricin NC8, plantaricin S and plantaricin W structural genes followed by DNA sequencing of amplicons were carried out to identify bacteriocin genes in *L. paraplantarum* FT259 genome. It was observed that *L. paraplantarum* FT259 tolerated exposure to pH 3.5, and bile salts 0.3 % for up to 180 minutes, but bacterial cells incubated at pH 2.0 and 2.5 for 90 minutes could not be enumerated (below the detection limit of the method, 2 log CFU/mL). In experiments with simulated gastric juice, viable cells of *L. paraplantarum* FT259 decreased from 8.6 log CFU/mL to 4.4 log CFU/mL after 180 minutes. Otherwise, *L. paracasei* FT700 survived well in almost all tested conditions. After 180 minutes at pH 2.0 or simulated gastric juice, bacterial populations were reduced by 4 and 3 log CFU/mL, respectively. It was also demonstrated that *L. paraplantarum* FT259 and *L. paracasei* FT700 were susceptible to the majority of the antibiotics tested. SDS-PAGE analysis indicated that the partially purified bacteriocin presented a molecular mass around 3,900 Da and plantaricin NC8 gene was detected by PCR and sequenced. Overall, the results indicated that both strains have potential probiotic traits, and the production of bacteriocins (FT259) or the proteolytic activity (FT700) may be interesting features for food applications.

Key Words: *Lactobacillus,* probiotic, dairy products, bacteriocin.

Avaliação preliminar do potencial probiótico de *Lactobacillus paraplantarum* FT259 e *Lactobacillus paracasei* FT700, isolados de queijos obtidos no Brasil

Resumo

Lactobacillus paraplantarum FT259 foi isolado a partir de queijo de vaca e produz bacteriocinas (capítulo 3). *Lactobacillus paracasei* FT700 foi isolado a partir de queijo de cabra e é capaz de produzir peptídeos com atividade imunomoduladora pela hidrólise de proteínas do leite durante a fermentação (capítulo 5). Este estudo teve como objetivo avaliar o potencial probiótico de *L. paraplantarum* FT259 (linhagem produtora de bacteriocina) e *L. paracasei* FT700 (linhagem proteolítica). As linhagens foram testadas quanto à sobrevivência em meio de cultura acidificado (pH 2,0, 2,5 e 3,5), tolerância *in vitro* aos sais biliares, viabilidade em suco gástrico sintético e sensibilidade a antibióticos. Além disso, os peptídeos antimicrobianos produzidos por *L. paraplantarum* FT259 foram parcialmente purificados em coluna com resina XAD-16, seguido por extração em fase sólida utilizando cartucho C_{18}. As bacteriocinas foram analisadas por eletroforese em gel de poliacrilamida com dodecil sulfato de sódio (*sodium dodecyl sulfate polyacrylamide gel electrophoresis*, SDS-PAGE). Reações da polimerase em cadeia (*polymerase chain reaction*, PCR) com *primers* para os genes estruturais das plantaricinas NC8, S e W, seguidas de sequenciamento de DNA, foram realizadas para a identificação de genes que codificam para a produção de bacteriocinas. Os resultados mostraram que *L. paraplantarum* FT259 sobreviveu em pH 3,5 e a 0,3 % de sais biliares por até 180 minutos, mas a população de células expostas aos pHs de 2,0 e 2,5 durante 90 minutos estava abaixo do limite de detecção do método (2 log UCF/mL). Nas análises com suco gástrico sintético, a população de *L. paraplantarum* FT259 diminuiu de 8,6 log UFC/mL para 4,4 log UFC/mL após 180 minutos. Entretanto, *L. paracasei* FT700 apresentou boa taxa de sobrevivência em quase todas as condições. Depois de 180 minutos em pH 2,0 e suco gástrico sintético, a população de células diminuiu 4 e 3 log UFC/mL, respectivamente. Também foi demonstrado que *L. paraplantarum* FT259 e *L. paracasei* FT700 foram sensíveis à maioria dos antibióticos testados. A análise por SDS-PAGE indicou que a bacteriocina parcialmente purificada apresenta massa molecular de aproximadamente 3900 Da, e por PCR seguido de sequenciamento de DNA foi detectada a presença do gene que codifica a produção da plantaricina NC8. De modo geral, os resultados indicaram que ambas as linhagens possuem potencial probiótico. Além disso, a produção de bacteriocinas (*L. paraplantarum* FT259) e a atividade proteolítica (*L. paracasei* FT700) podem ser características interessantes para aplicações desses micro-organismos em alimentos.

Palavras-chave: *Lactobacillus*, probióticos, produtos lácteos, bacteriocina.

Évaluation préliminaire du potentiel probiotique de *Lactobacillus paraplantarum* FT259 et de *Lactobacillus paracasei* FT700, isolés de fromages brésiliens

Résumé

Lactobacillus paraplantarum FT259 (producteur d'une bactérioine) a été isolé d'un fromage de vache (chapitre 3). *Lactobacillus paracasei* FT700, dont la fermentation du lait produit des peptides immonomodulateurs, comme il a été démontré dans le chapitre 5, a été quant à lui isolé d'un fromage de chèvre. Cette étude visait à évaluer le potentiel probiotique de ces deux souches, *L. paraplantarum* FT259 et *L. paracasei* FT700. La capacité de survie des souches a été testée *in vitro* dans des milieux de culture acidifiée (pH 2.0, 2.5 et 3.5), en présence de sels biliaires, ainsi que dans des conditions simulant les conditions gastriques, leur sensibilité aux antibiotiques a également été déterminée. En outre, les peptides antimicrobiens produits par *L. paraplantarum* FT259 ont été partiellement purifiés dans une colonne contenant une résine XAD-16, suivie d'une extraction en phase solide avec une cartouche C_{18}, suivie d'une électrophorèse sur gel de polyacrylamide en condition dénaturante (*sodium dodecyl sulfate polyacrylamide gel electrophoresis*, SDS-PAGE). Des PCR (*polymerase chain reaction*) avec les amorces ciblant les gènes structurels de la plantaricine NC8, de la plantaricine S et de la plantaricine W ont été effectuées, ainsi que le séquençage des amplicons afin d'identifier les gènes codant pour les bactériocines. Les résultats ont montré que *L. paraplantarum* FT259 tolérait une exposition à un pH de 3,5, et à une concentration en sels biliaires de 0,3% pendant une durée maximum de 180 minutes. En revanche, il n'était plus possible de détecter de cellules viables (limite de détection de la méthode: 2 log UFC/mL) après une exposition à des pH de 2,0 et de 2,5 pendant 90 minutes. Lors des expériences simulant les conditions gastriques, le nombre de cellules viables de *L. paraplantarum* FT259 a diminué de 8,6 log UFC/mL à 4,4 log UFC/mL après 180 minutes d'incubation. Les résultats obtenus pour la souche *L. paracasei* FT700 ont montré que celle-ci a survécu à presque toutes les conditions testées. Après 180 minutes d'incubation à un pH de 2,0 et dans les conditions gastriques, les populations bactériennes ont respectivement diminué de 4 et de 3 log UFC/mL. Il a également été démontré que *L. paraplantarum* FT259 et *L. paracasei* FT700 étaient sensibles à la majorité des antibiotiques testés. L'analyse par SDS-PAGE a montré que la bactériocine partiellement purifiée présentait une masse moléculaire d'environ 3900 Da et le séquençage des amplicons d'ADN suite aux PCR a montré qu'il possedáit le gène de la plantaricine NC8. L'ensemble de ces résultats indique que les deux souches de lactobacilles isolées lors de cette étude (*L. paraplantarum* FT259 et *L. paracasei* FT700) possèdent des caractéristiques pouvant leur conférer un intérêt en tant que probiotiques. La production de bactériocines (*L. paraplantarum* FT259) et l'activité protéolytique (*L. paracasei* FT700) pourraient également être des caractéristiques intéressantes pour des applications alimentaires. En conclusion, les BAL obtenues dans cette étude pourraient être utiles dans l'industrie alimentaire, soit pour la production de produits laitiers à durée de conservation prolongée ou possédant une digestibilité accrue, soit pour la production de peptides bioactifs.

Mots clés: *Lactobacillus*, probiotiques, des produi ts laitiers, des bactériocines.

1. Introduction

To qualify a microorganism as probiotic, certain criteria have to be fulfilled: (i) identification at genus, species, and strain level, (ii) production of antimicrobial substances (e. g. bacteriocins), (iii) safety for food and clinical use, (iv) survival during gastrointestinal passage, (v) adhesion to mucosal tissue, (vi) colonization of the human intestine (at least temporarily), (vii) inhibition of pathogenic bacteria, (viii) clinically documented and validated health effect and (ix) stability during processing and storage (BORCHERS et al., 2009; FOOD AND AGRICULTURE ORGANIZATION; WORLD HEALTH ORGANIZATION, 2002; SAAD et al., 2013; VERNA; LUCAK, 2010;). In this study, some of these criteria were evaluated for the bacteriocin producer *Lactobacillus paraplantarum* FT259, and for the proteolytic *Lactobacillus paracasei* FT700, both isolated from Brazilian cheeses. In addition, the bacteriocin produced by the former strain was partially purified and characterized, and the genes encoding for bacteriocin production were determined. Some of the results presented in this chapter were published by Tulini, Winkelströter and De Martinis (2013).

2. Materials and methods

2.1. Bacterial strains

L. paraplantarum FT259 was isolated from Brazilian cow cheese and it was chosen for this study due to its antilisterial activity (chapter 3). Likewise, *L. paracasei* FT700 was isolated from Brazilian goat cheese and it was chosen for this study due to its proteolytic activity, associated with the production of immunomodulatory peptides during milk fermentation (chapter 5). Both strains were identified in a previous work by *16S rRNA* gene sequencing and PCR with species-specific primers. The strains were grown at 37 °C in MRS broth (De Man, Rogosa, Sharpe, Oxoid, UK). In all assay done with anaerobic incubation, the atmosphere generation system AnaeroGen™ (Oxoid) was used. *Listeria monocytogenes* IAL 633 was chosen as indicator strain for antagonistic tests and it was cultured at 37 °C for 24 h in BHI broth (Brain Heart Infusion, Oxoid). The strains were maintained at -80 °C in BHI or MRS broth for *L. monocytogenes* and lactobacilli, respectively, containing 20 % v/v glycerol (Synth, Brazil).

2.2 Evaluation of probiotic traits

2.2.1 Acid tolerance

Lactobacilli were evaluated for survival at low pH, as described by Maragkoudakis et al. (2006), with modifications. Briefly, 1 mL of an overnight lactobacilli culture in MRS broth at 37 °C was centrifuged for 5 minutes at 10,000 x *g*. The cell pellet was resuspended with 1 mL of MRS broth with pH previously adjusted to 2.0, 2.5 or 3.5, using 4 M HCl. The bacterial suspension was incubated at 37 °C for 0, 90 and 180 minutes. Enumeration of bacterial population (CFU/mL) was done by surface plating on MRS agar (100 µL) followed by incubation at 37 °C for 48 h under anaerobic atmosphere.

2.2.2 *In vitro* survival in simulated gastric juice

Lactobacilli were challenged to survive in simulated gastric juice as described by Gomes et al. (2012), with modifications. Simulated gastric juice was prepared by adding 3.0 g/L of pepsin (Sigma-Aldrich, USA) in 0.85 % w/v NaCl solution, adjusted to pH 2.0 with 4 M HCl. One milliliter of an overnight lactobacilli culture in MRS broth at 37 °C was centrifuged for 5 minutes at 10,000 x *g*, and the cell pellet was resuspended with the same volume of simulated gastric juice. The bacterial suspension was kept at 37°C for 0, 90 and 180 minutes. Enumeration of bacterial population (CFU/mL) was done by surface plating on MRS agar (100 µL) followed by incubation at 37 °C for 48 h under anaerobic atmosphere.

2.2.3 Tolerance to bile salts

One milliliter of an overnight lactobacilli culture in MRS broth was centrifuged for 5 minutes at 10,000 x *g*. The cell pellet was resuspended with 1 mL of MRS broth added of 0.3 % w/v bile salts (Oxgall, Sigma-Aldrich) and kept at 37 °C for 0, 90 and 180 minutes. Enumeration of bacterial population was done by surface plating on MRS agar (100 µL) followed by incubation at 37 °C for 48 h under anaerobic

atmosphere (GOMES et al., 2012; MARAGKOUDAKIS et al., 2006; THIRABUNYANON; BOONPRASOM; NIAMSUP, 2009).

2.2.4 Antibiotic susceptibility test

Antibiotic susceptibility profile was determined by disc diffusion assay using a modification of the agar overlay diffusion method, as described by Charteris et al. (1998) and Wang et al. (2010). MRS agar plates were overlaid with 4 mL of MRS soft agar (0.8 % w/v bacteriological agar, Oxoid) inoculated with 200 µL (*ca.* 10^6 CFU) of an overnight lactobacilli culture grown in MRS broth at 37 °C. Antibiotic discs (Laborclin, Brazil) containing ampicillin (10 µg), cephalexin (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), erythromycin (15 µg), gentamicin (10 µg), penicillin G (10 U), rifampicin (5 µg), tetracycline (30 µg) and vancomycin (30 µg) were placed on plates surface and incubated at 37 °C for 24 h under anaerobic atmosphere. Inhibition zones were measured and the lactic acid bacteria (LAB) strain was scored as resistant, moderately susceptible and susceptible, according to the cut-off level proposed by Charteris et al. (1998). The strains were considered resistant if the inhibition halo was smaller than 12 mm for ampicillin, 15 mm for cephalexin, 13 mm for chloramphenicol, 13 mm for ciprofloxacin, 8 mm for clindamycin, 13 mm for erythromycin, 12 mm for gentamicin, 19 mm for penicillin G, 14 mm for rifampicin, 14 mm for tetracycline and 14 mm for vancomycin.

2.3 Partial purification and characterization of bacteriocin produced by *L. paraplantarum* FT259

2.3.1 Bacteriocin purification

Bacteriocin purification was performed according to Martin-Visscher et al. (2008) with some modifications. For that, *L. paraplantarum* FT259 was inoculated at 10^6 CFU/mL in 1,400 mL of MRS broth and incubated at 37 °C for 24 h. The culture supernatant was obtained by centrifugation at 14,000 x *g* for 10 min at 4 °C. The cell-free supernatant was applied to a glass column (25 x 500 mm, Bio-rad Laboratories, USA) filled with 60 g of Amberlite XAD-16 resin (Sigma-Aldrich) at a flow rate of 8 mL/min. The column was washed with 700 mL of purified water, followed by 700 mL

of 30 % v/v ethanol (Synth), at a flow rate of 12 mL/min. The active fraction was eluted with 700 mL of 70 % v/v isopropyl alcohol (JT Baker, USA) acidified to pH 2.0 with 4 M HCl, at a flow rate of 12 mL/min. The alcohol was eliminated using a rotary evaporator (Fisatom, Brazil), and the sample was loaded onto a solid phase extraction cartridge (HF Mega Bond Elut C_{18}, 10 g - 60 mL, Varian, USA), previously conditioned with methanol. The cartridge was washed with 60 mL of purified water, 60 mL of 30 % v/v ethanol, 60 mL of 20 % v/v isopropyl alcohol and 60 mL of 40 % v/v isopropyl alcohol. The bacteriocins were eluted with 60 mL of 70 % v/v isopropyl alcohol acidified to pH 2.0 with 4 M HCl. Isopropyl alcohol was removed on a rotatory evaporator followed by freeze-drying (Liotop L202, Liobrás, Brazil).

2.3.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The samples containing the bacteriocins were analyzed in a vertical electrophoresis system (Mini-PROTEAN II Electrophoresis Cell, Bio-rad Laboratories) based on the protocol proposed by El-Ghaish et al. (2010), with modifications. Polyacrylamide gel composition is described in Table 6.1. The sample buffer contained 4 % w/v SDS (sodium dodecyl sulfate, Synth), 3 % v/v mercaptoethanol (Sigma-Aldrich), 10 % v/v glycerol (Synth), 50 mM tris-HCl pH 6.8 (Serva, Germany) and 0.07 % w/v bromophenol blue (Bio-Agency, Brazil). The partially purified bacteriocins diluted in sample buffer were heated at 100 °C for 3 minutes and analyzed by SDS-PAGE, along with pre-stained low molecular weight marker (3,500 to 38,000 Da, GE Life Sciences, Sweden). The running buffer was prepared with 50 mM tris base (Serva), 0.384 M glycine (Sigma-Aldrich) and 0.1 % w/v SDS (Synth), and the electrophoretic separation was carried out with 10 mA for 90 minutes.

Table 6.1 Composition of polyacrylamide gels used for SDS-PAGE analyses of partially purified bacteriocins. Values are presented for plates with 0.75 mm spacers

Component	Staking gel (5 %)	Resolving gel (16 %)
Acrylamide-bis[1] (29:1) 40 %	625 µL	4000 µL
Tris-HCl[2] 2 M, pH 8.8	0	1665 µL
Tris-HCl[2] 0.5 M, pH 6.8	600 µL	0
Water	3677 µL	4115 µL
SDS[3] 10 %	50 µL	100 µL
TEMED[4]	8 µL	20 µL
Ammonium persulfate[2] 10 % (aqueous solution)	40 µL	100 µL
Total	**5000 µL**	**10000 µL**

[1] Supplier: Sigma-Aldrich, USA.
[2] Supplier: Synth, Brazil.
[3] Sodium dodecyl sulfate (Synth).
[4] N,N,N′,N′-Tetramethylethane-1,2-diamine (Sigma-Aldrich).

Samples were applied in duplicate in the gels, and they were revealed separately by silver staining and using a biological indicator, according to Schägger (2006) and Tulini and De Martinis (2010), with modifications. Silver staining was carried out on half of the gel, as described in Table 6.2, and the other half was revealed with a biological indicator. The latter was fixed with an aqueous solution made of ethanol (40 % v/v, Synth) and glacial acetic acid (10 % v/v, Synth) for 30 minutes, and rinsed with purified water for 2 h. The gel was placed on a BHI agar plate, and overlaid with 7 mL of soft-agar BHI (0.8 % w/v agar) seeded with *L. monocytogenes* IAL 633 (*ca.* 10^6 CFU/mL), followed by incubation at 37 °C for 24 h. The occurrence of inhibition halo was indicative of bacteriocin activity, and the biological revealed side of the gel was compared with silver stained gel.

Table 6.2 Silver staining method used to stain polyacrylamide gels after SDS-PAGE analysis of partially purified bacteriocins, obtained by purification with XAD-16 resin, followed by solid phase extraction in a C_{18} cartridge[1]

Step	Solution	Volume	Time
1. Fixation	Absolute ethanol[2]	100 mL	30
	Glacial acetic acid[2]	25 mL	minutes
	Add water to final volume	250 mL	
2. Washing	Purified water	-	2 x 30
			minutes
3. Sensitization	Sodium thiosulfate[3] (5 % w/v)	0.25 mL	30
	Add water to final volume	250 mL	minutes
4. Reaction with	Silver nitrate[4] (2.5 % w/v)	10mL	30
silver	Add water to final volume	250mL	minutes
5. Washing	Purified water	-	30
			seconds
6. Developing	Sodium carbonate[4]	5 g	~ 3 - 5
	Formaldehyde[4] (37 % w/v)	240 µL	minutes
	Add water to final volume	250 mL	
7. Stopping	EDTA-$Na_2(2H_2O)$[3]	3.65 g	30
	Add water to final volume	250 mL	minutes
8. Washing	Purified water	-	3 x 5
			minutes

[1] Schägger (2006) with modifications.
[2] Supplier: Synth, Brazil.
[3] Supplier: Merck, Germany.
[4] Supplier: Sigma-Aldrich, USA.

2.3.3 Bacteriocin structural gene amplification and DNA sequencing

The genomic DNA of *L. paraplantarum* FT259 was obtained with the Illustra bacteria genomic Prep Mini Spin Kit (GE Life Sciences) and used to detect bacteriocin structural genes. Polymerase chain reaction (PCR) was carried out with 8.4 µL of Taq Platinum Blue (Invitrogen, USA), 3 pmol of each primer (forward and reverse) and 30 ng of target DNA (10 µL of final volume for the reaction mixture). Bacteriocin genes were amplified with primers targeting plantaricin NC8, plantaricin S and plantaricin W structural genes (HOLO et al., 2001; MALDONADO; RUIZ-BARBA;

JÍMENEZ-DÍAZ, 2003; STEPHENS et al., 1998), as presented in Table 6.3. The thermal cycling used was: denaturation at 94 °C for 1 minute, annealing at suitable temperature (see Table 6.3) for 1 minute, and extension at 72 °C for 30 seconds, in 30 cycles. The PCR products were analyzed in 1 % w/v agarose gel and purified using Illustra GFX PCR DNA and Gel Band Purification (GE Life Sciences). PCR products were sequenced using ABI 3730 DNA Analyzer (Applied Biosystems, USA) with BigDye Terminator v3.1 Cycle Sequencing Reagent (Applied Biosystems) at the Human Genome Research Center (HGRC) of the University of São Paulo, Brazil. Sequences were analyzed using Chromas Lite 2.1 (Technelysium, Australia) and compared with sequences available in GenBank, with the National Center for Biotechnology Information BLASTN search program (http://www.ncbi.nlm.nih.gov/BLAST).

Table 6.3 Oligonucleotide sequences of the primers used in this study to detect genetic determinants of bacteriocin production

Target bacteriocin	Oligonucleotide sequence (5'-3')	Annealing temperature (°C)	Reference
Plantaricin NC8	Fw: GGT CTG CGT ATA AGC ATC GC Rv: AAA TTG AAC ATA TGG GTG CTT TAA ATT CC	60	MALDONADO; RUIZ-BARBA; JÍMENEZ-DÍAZ, 2003
Plantaricin S	Fw: GCC TTA CCA GCG TAA TGC CC Rv: CTG GTG ATG CAA TCG TTA GTT T	60	STEPHENS et al., 1998
Plantaricin W	Fw: TCA CAC GAA ATA TTC CA Rv: GGC AAG CGT AAG AAA TAA ATG AG	55	HOLO et al., 2001

2.4 Statistical analysis

All experiments were performed as biological triplicates and statistical analysis of results was done with GraphPad Prism software (GraphPad, USA). Analysis of

variance (ANOVA) followed by Bonferroni test was applied to detect significant differences (P<0.05).

3. Results and discussion

3.1 Probiotic traits evaluation

3.1.1 Acid tolerance

The viability and survival of bacteria in acidic environment is one of the most important parameters for establishing probiotic properties. The pH of the stomach generally ranges from 2.5 to 3.5 and this is a barrier against the entry of bacteria into the intestinal tract (BOKE; ASLIM; ALP, 2010; LIN et al., 2007). In the present paper, *L. paraplantarum* FT259 and *L. paracasei* FT700 were challenged at low pH in MRS broth and initial counts ranged from *ca.* 7.5 to 8.5 log CFU/mL, at pH 2.0, 2.5 and 3.5 (Figures 6.1 and 6.2).

For *L. paraplantarum* FT259, after 90 minutes and 180 minutes of incubation, bacterial population were below the detection limit of the method (< 2 log CFU/mL), at pH 2.0 and 2.5. However, at pH 3.5, bacterial population decreased to 7.7 ± 0.1 log CFU/mL after 180 minutes. *L. paracasei* FT700 survived at low pH, with a four log CFU/mL reduction observed at pH 2.0 after 180 minutes of incubation, while no statistically significant decrease on bacterial population was detected in higher pH. Cebeci and Gurakan (2003) reported good survival rates of lactobacilli after 90 minutes in MRS broth adjusted to pH 3.5, similar to the present results.

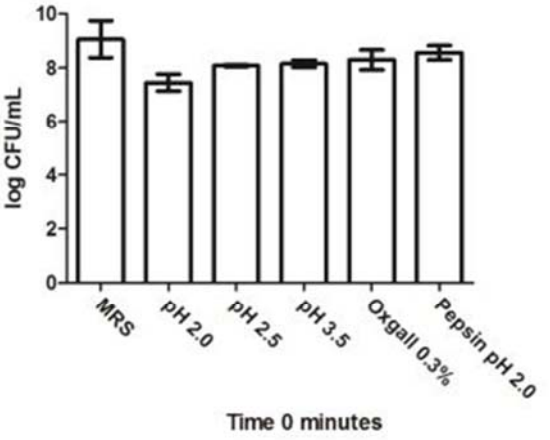
3.1.2 Survival in simulated gastric juice

It is desirable to screen potential probiotics candidates for their capacity to tolerate conditions of the upper gastrointestinal tract (BOKE; ASLIM; ALP, 2010). *L. paraplantarum* FT259 initial population was 8.6 log CFU/mL and it dropped to 5.9 log CFU/mL and 4.4 log CFU/mL, respectively, after 90 and 180 minutes. Similarly, *L. paracasei* FT700 initial population reduced from 8.7 log CFU/mL to 6.1 log CFU/mL and 5.9 CFU/mL, respectively, after 90 and 180 minutes. Results are presented in Figures 6.1 and 6.2.

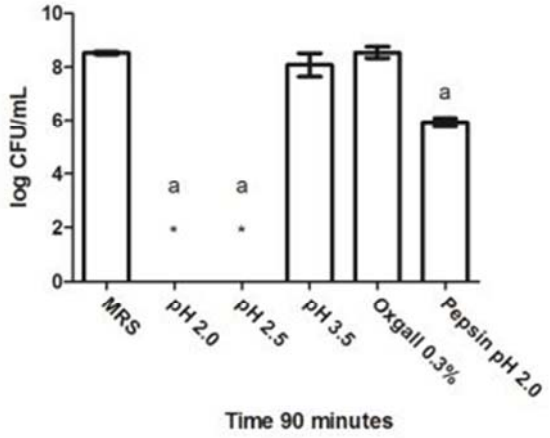
Maragkoudakis et al. (2006) examined the probiotic potential of twenty-nine lactobacilli strains of dairy origin and they reported significant bacterial population reduction under simulated gastrointestinal tract conditions. Lee et al. (2011) reported similar results for *Lactobacillus* spp. and affirmed that only 118 out of 350 strains tested survived after 2 h of incubation in the presence of pepsin at pH 2.5. Otherwise, a considerable resistance to simulated gastric juice for a *L. paracasei* strain was observed by Wang et al. (2010). Those authors evaluated the resistance of a *L. paracasei* in pH 2.0 added of pepsin for 180 minutes and reported only one log CFU/mL reduction on the initial bacterial population. These data reinforce the survival of lactobacilli in gastric conditions may vary greatly among different species and strains.

3.1.3 Tolerance to bile salts

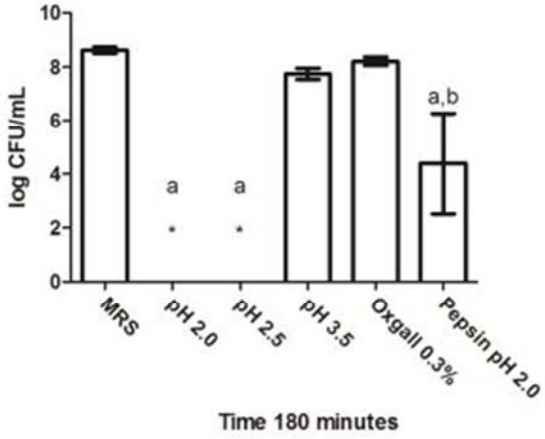
Bile salts act *in vivo* as biological detergents that emulsify and solubilize lipids, thereby playing an essential role in fat digestion (BEGLEY; HILL; GAHAN, 2006). Human bile juice does not match exactly the composition of 0.3 % Oxgall solution used in this research, but this compound is frequently used to evaluate the viability of probiotic candidate strains (GOMES et al., 2012; LIN et al., 2007; MARAGKOUDAKIS et al., 2006). In 2009, Thirabunyanon, Boonprasom and Niamsup evaluated the survival of LAB dairy isolates in 0.3 % bile salts medium. Those authors reported LAB strains survived well in such conditions. In this study, similar results were also observed, as both lactobacilli were not affected by exposure to bile salts (Figures 6.1 and 6.2).



A)

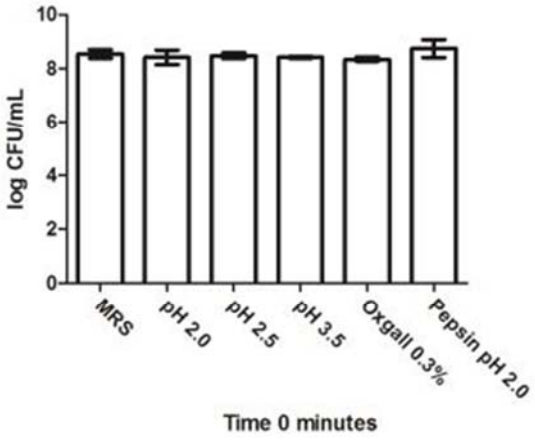


B)

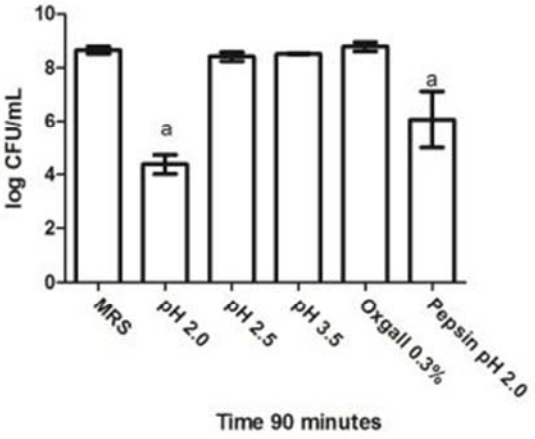


C)

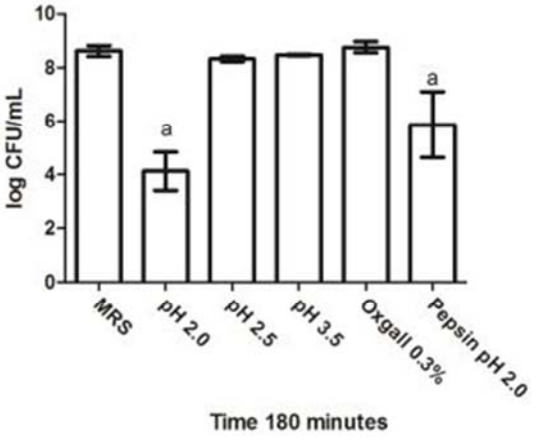
Figure 6.1 Survival of *Lactobacillus paraplantarum FT259* exposed to low pH (2.0, 2.5 and 3.5), bile salts (Oxgall 0.3 %) and simulated gastric juice (pepsin, pH 2.0) followed by incubation for 0 (A), 90 (B) and 180 (C) minutes at 37 °C. “a” - statistically different from 0 minutes (P<0.05); “b” – statistically different from 90 minutes (P<0.05). Data represent the mean of three independent replicates ± standard deviation. * Population below detection limit of the method (2 log CFU/mL).



A)



B)



C)

Figure 6.2 Survival of *Lactobacillus paracasei* FT700 exposed to low pH (2.0, 2.5 and 3.5), bile salts (Oxgall 0.3 %) and simulated gastric juice (pepsin, pH 2.0) followed by incubation for 0 (A), 90 (B) and 180 (C) minutes at 37 °C. "a" - statistically different from 0 minutes (P<0.05). Data represent the mean of three independent replicates ± standard deviation. * Population below detection limit of the method (2 log CFU/mL).

3.1.4 Antibiotic susceptibility

To avoid the transfer of genetic resistance elements to intestinal human microbiota, it is important to screen probiotic strains for this trait (ARICI et al., 2004; BORRIELLO et al., 2003; OCAÑA; SILVA; NADER-MACIAS, 2006; TINRAT; SARAYA; TRAIDEJ CHOMNAWANG, 2011). In this study, *L. paraplantarum* FT259 and *L. paracasei* FT700 were tested using antibiotics belonging to different groups: (i) inhibitors of cell wall synthesis (penicillin G, ampicillin, cephalexin and vancomycin); (ii) inhibitors of protein synthesis (gentamicin, tetracycline, chloramphenicol, erythromycin and clindamycin) and (iii) inhibitors of nucleic acid synthesis (ciprofloxacin and rifampicin). Both strains were resistant to vancomycin and ciprofloxacin, but they were susceptible to the all other antibiotics (ampicillin, cephalexin, chloramphenicol, clindamycin, erythromycin, gentamicin, penicillin G, rifampicin and tetracycline). According to Nelson (1999), the genera *Leuconostoc*, *Pediococcus* and *Lactobacillus* are intrinsically resistant to glycopeptides such as vancomycin, and the intrinsic resistance to ciprofloxacin is a common feature among LAB (HUMMEL et al., 2007). In addition, *L. paraplantarum* FT259 and *L. paracasei* FT700 showed the same antibiotic resistance profile as the lactobacilli evaluated by Arici et al. (2004) and Xanthopoulos, Litopoulou-Tzanetaki and Tzanetakis (2000). These data with respect to antibiotic resistance profiles suggest the safety of *L. paraplantarum* FT259 and *L. paracasei* FT700 as potential probiotic candidates.

3.2.3 Partial purification and characterization of bacteriocins produced by *L. paraplantarum* FT259

3.2.3.1 Bacteriocin purification and analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

LAB have the ability to produce a variety of antimicrobial metabolites, including bacteriocins, which are peptides are usually active against Gram-positive bacteria, such as *L. monocytogenes* (GARCÍA-ALMENDÁREZ et al., 2008; SIT and VEDERAS, 2008).

To the best of our knowledge, only one bacteriocin produced by this species has been characterized (LEE et al., 2007), the paraplantaricin C7. This bacteriocin

was described as a class II bacteriocin produced by *L. paraplantarum* C7 (molecular mass *ca.* 3,800 Da), according to the classification proposed by Cotter, Hill and Ross (2005) and KLAENHAMMER (1994).

In the present study, the partially purified bacteriocin produced by *L. paraplantarum* FT259 was analyzed by SDS-PAGE, and it presented a molecular mass around 3,900 Da (Figure 6.3) which is compatible with the small and thermostable classes I or II bacteriocins and with the results obtained by PCR, described as follows (COTTER, HILL and ROSS, 2005; KLAENHAMMER, 1994).

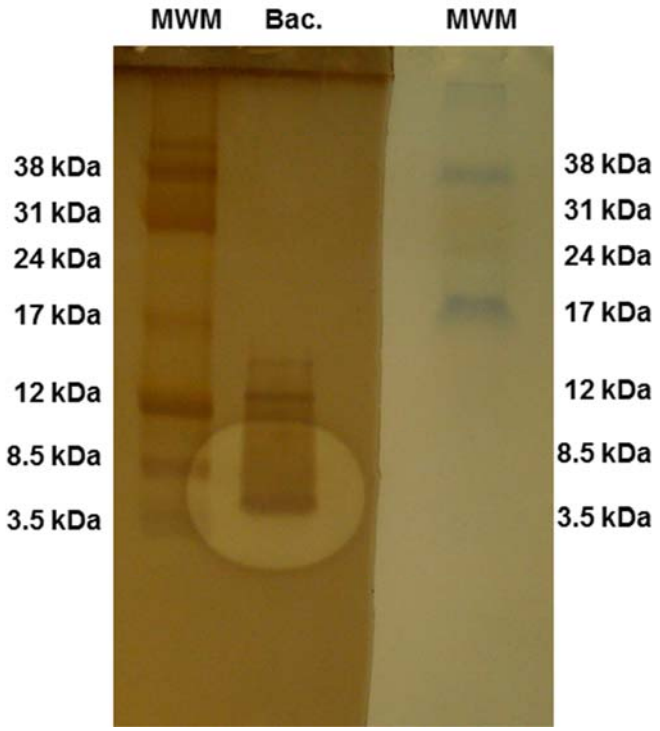


Figure 6.3 Photography of superposed gels from SDS-PAGE analysis of partially purified bacteriocins produced by *Lactobacillus paraplantarum* FT259. On the left side, silver stained gel. On the right side, gel revealed with the biological indicator *Listeria monocytogenes* showing an inhibition halo in the same area where the smallest peptide (*ca.* 3900 Da) was detected in silver-stained gel, corresponding to the bacteriocin. MWM: molecular weight marker; Bac.: partially purified bacteriocin.

3.2.3.2 Bacteriocin structural gene amplification and DNA sequencing

To identify genetic determinants responsible for bacteriocin production by *L. paraplantarum* FT259, primers targeting plantaricins NC8, S and W structural genes were used for PCR analysis. However, only plantaricin NC8 structural gene was detected. DNA sequencing of the PCR product (Figure 6.4) revealed a gene

sequence similar to that encoding for plantaricin NC8α in *Lactobacillus plantarum* NC8 (MALDONADO; RUIZ-BARBA; JÍMENEZ-DÍAZ, 2003), except for two different nucleotides in the sequence that codifies the leader peptide (position 91 and 117), and one in the sequence that codifies the mature peptide (position 132). These mutations did not affect the predicted mature active peptide, but in the predicted leader peptide, a tyrosine residue would be replaced by an alanine residue.

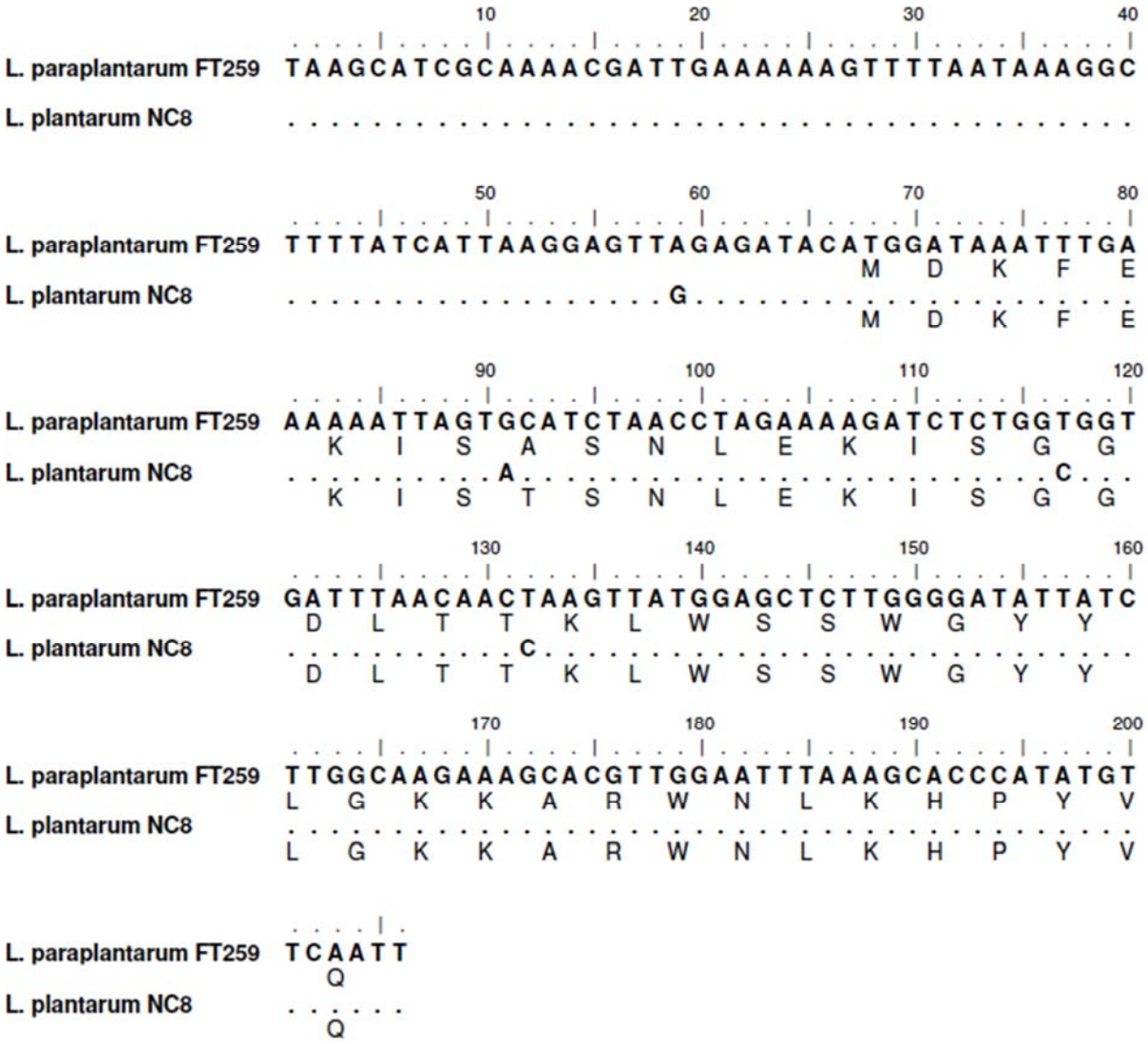


Figure 6.4 Gene sequence and predicted amino acid sequence obtained by the amplification of plantaricin NC8 structural gene, using *Lactobacillus paraplantarum* FT259 genomic DNA and primers plnNC8-f/plnNC8-r. The bacteriocin gene sequence was compared with the one codified by *Lactobacillus plantarum* NC8. Sequences were analyzed and aligned using Chromas Lite 2.1 program (Technelysium, Australia). Dots represent identical nucleotides.

Maldonado, Ruiz-Barba and Jímenez-Díaz (2003) were the first to describe the plantaricin NC8, which is a two-peptide bacteriocin composed by plantaricin

NC8α (3,587 Da) and plantaricin NC8β (4,000 Da), both important for full bacteriocin activity. Although only plantaricin NC8α structural gene was evaluated in the present study, it is possible that *L. paraplantarum* FT259 also harbors the plantaricin NC8β structural gene, since antimicrobial activity was detected. The results of DNA sequencing corroborate the data obtained by SDS-PAGE, indicating a molecular mass of *ca.* 3,900 Da, similar to the one described for plantaricin NC8α and NC8β, which is, respectively, 3,587 Da and 4,000Da. In 2011, Hurtado et al. used the same primers described in Table 6.3 to search plantaricin genes (NC8, S and W) in ninety eight isolates of *L. paraplantarum*, *Lactobacillus pentosus* and *L. plantarum*. Those authors obtained amplicons from *L. paraplantarum* and *L. pentosus* isolates with at least 97 % of homology with published sequences for *L. plantarum*, and detected plantaricin NC8 structural gene in 16.7 % of *L. paraplantarum* isolates.

4. Conclusions

The cheese isolates *L. paraplantarum* FT259 and *L. paracasei* FT700 tolerated selected simulated gastrointestinal conditions and they were susceptible to the majority of the antibiotics tested. In addition, the bacteriocin produced by *L. paraplantarum* FT259 was partially purified and characterized, with results indicating this strain harbors the plantaricin NC8 structural gene, and produces a bacteriocin with molecular mass of *ca.* 3,900 Da. *L. paraplantarum* FT259 and *L. paracasei* FT700 are good candidates as probiotic and/or biopreservative strains, and they deserve further investigation to elucidate the potential health benefits and applications in food.

7. Closing remarks

- In this study, 156 samples of cow, buffalo and goat milk were analyzed to isolate LAB with antimicrobial or proteolytic activity. The isolates with interesting properties were identified by *16S rRNA* gene sequencing.

- Four LAB produced bacteriocins, and they were identified as *Streptococcus uberis* FT86 (isolated from cow milk), *S. uberis* FT126 (isolated from cow milk), *S. uberis* FT190 (isolated from cow milk) and *Lactobacillus paraplantarum* FT259 (isolated from cow cheese).

- *L. paraplantarum* FT259 presents the gene for the production of plantaricin NC8, and SDS-PAGE showed the antimicrobial peptide produced by this strain has *ca*. 3,900 Da, similar to one of the units of plantaricin NC8, previously described in the literature. So, it is very likely *L. paraplantarum* FT259 produces the plantaricin NC8.

- In addition, *L. paraplantarum* FT259 presented probiotic potential, since it survived at in acidic pH and in the presence of bile salts, which shows that this strain has high potential to be used in food products.

- Two strains (*Weissella confusa* FT424 and *Lactobacillus plantarum* FT723) presented antifungal activity toward *Penicillium expansum*. Moreover, *L. plantarum* FT723 inhibited *P. expansum* in a fermented milk model, indicating the potential of this strain for preservation of dairy products.

- *Enterococcus faecalis* FT132 and *Lactobacillus paracasei* FT700 were proteolytic in milk with the best activity at pH 6.5 and in the range of 37 to 42 °C.

- Fermented milk supernatant produced by *E. faecalis* FT132 and *L. paracasei* FT700 stimulated the differentiation of monocytes into macrophages with low production of TNF-α (no inflammatory process). This suggests that beneficial immune response could be stimulated by the hydrolyzed milk proteins, which could be obtained by the production of fermented dairy products.

- *E. faecalis* FT132 harbored the virulence genes *asa*1, *ace* and *gel*E, it was resistant to erythromycin and tetracycline, discouraging its application in food. However, the

purified peptides derived from milk protein hydrolysis by this LAB may be further evaluated for food applications.

- *L. paracasei* FT700 presented probiotic potential due to survival at pH 2.0 and tolerance to bile salts. This is strain was not resistant to any of the antibiotics tested, except for those of intrinsic resistance (vancomycin and ciprofloxacin). *L. paracasei* has the potential to be used to produce a fermented drink with probiotic benefits and immune system stimulatory properties.

- In summary, it was isolated several LAB from milk and cheese samples that could be candidates for production of bioactive compounds and development of new dairy products with increased shelf-life and health promoting claims.

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Dissemination of research results

Poster presentation

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Partial purification and characterization of a bacteriocin produced by *Lactobacillus paraplantarum* FT259, isolated from Brazilian semi-hard artisanal cheese

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INTRODUCTION

Bacteriocins (antimicrobial peptides synthesized by bacteria) are of interest in the food industry to control food borne pathogens such as *Listeria monocytogenes*. These peptides can be degraded by digestive enzymes, representing a potentially safe alternative to classical food preservatives. The bacteriocinogenic *Lactobacillus paraplantarum* FT259 has probiotic potential, as demonstrated by previous studies[(1)], and this stimulates researches on the antimicrobial peptide produced.

PURPOSE

The aim of this study was to partially purify and characterize the bacteriocin produced by *L. paraplantarum* FT259.

METHODS

1. Bacteriocin partial purification [(2)]

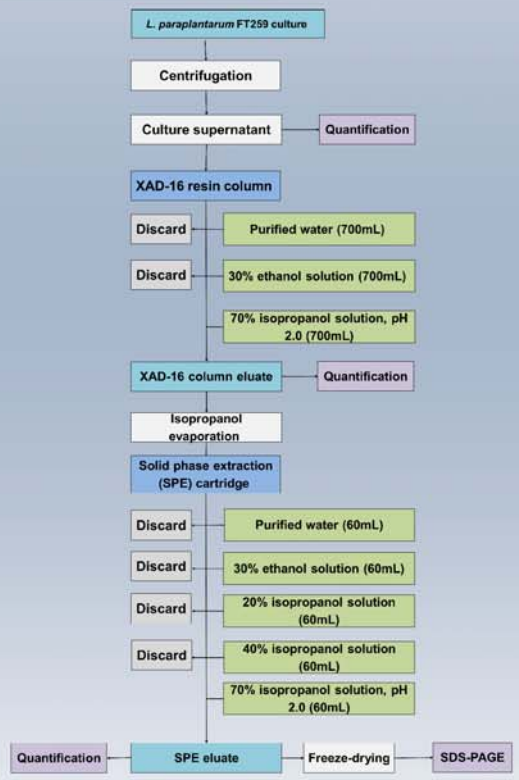

Figure 1. Flowchart of the purification of bacteriocins produced by *L. paraplantarum* FT259 with XAD-16 resin followed by solid phase extraction. Bacteriocin titres were measured by critical dilution assay.

2. Detection of genes related to plantaricin production [(3)]

Genomic DNA was obtained using Illustra bacteria genomicPrep mini spin kit (GE Healthcare, UK) and polymerase chain reaction (PCR) was carried out to detect genes related to plantaricin production (*plan*A, *plan*B, *plan*C, *plan*D, *plan*EF, *plan*G, *plan*I, *plan*J, *plan*K, *plan*N, *plan*NC8, *plan*S, *plan*W). Amplicons were analysed in agarose gel electrophoresis and stained with ethidium bromide.

The PCR products of plantaricin structural genes were purified using Illustra GFX PCR DNA and Gel Band Purification (GE Life Sciences). They were sequenced using ABI 3730 DNA Analyzer (Applied Biosystems, USA) with BigDye Terminator v3.1 Cycle Sequencing Reagent (Applied Biosystems) and compared with sequences available in GenBank, with the National Center for Biotechnology Information BLASTN search program (http://www.ncbi.nlm.nih.gov/BLAST).

RESULTS

The partially purified bacteriocin produced by *L. paraplantarum* FT259 was obtained with an yield of 45.7% (1,024,000 AU in 20 mL of SPE eluate). By SDS-PAGE, the molecular weight of the bacteriocin was estimated to be around 3,900 Da (Figure 2).

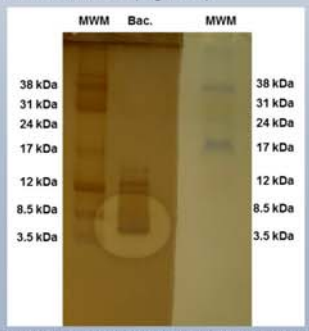

Figure 2. Superposed gels of SDS-PAGE analysis of partially purified bacteriocin produced by *L. paraplantarum* FT259. On the left side, silver stained gel. On the right side, gel revealed with the biological indicator *L. monocytogenes*. MWM: molecular weight marker; Bac.: partially purified bacteriocin.

Only the cluster *plan*ABCD (signal transduction pathway) and the plantaricin NC8 structural gene were detected by PCR, suggesting the production of plantaricin NC8 by this strain. Figure 3 presentes the sequence of plantaricin NC8 gene detected in *L. paraplantarum* FT259, in comparison to *L. plantarum* NC8 sequence availabe at GenBank database.

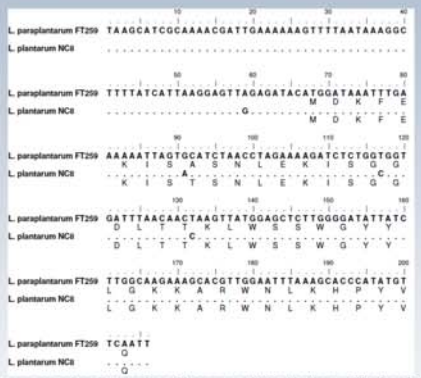

Figure 3. Gene sequence and predicted amino acid sequence obtained by the amplification of plantaricin NC8 structural gene, using *L. paraplantarum* FT259 genomic DNA and primers plNC8-f/plNC8-r[(3)]. The sequence was compared with the one codified by *L. plantarum* NC8. Dots represent identical nucleic acids.

SIGNIFICANCE

Characterization of the bacteriocin produced by *L. paraplantarum* FT259, as well as its purification process and the knowledge of genes related to bacteriocin production will help to design strategies for its biotechnological application.

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Evaluation of the proteolytic activity, virulence genes and antibiotic resistance of *Enterococcus faecalis* FT132, isolated from Brazilian raw cow milk

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INTRODUCTION

Milk is of great importance for human nutrition. However, it can be a problem for people allergic to its constituents, such as caseins and lactoserum proteins. The role of lactic acid bacteria in the modification of milk proteins during fermentation processes and their role in production of peptides with different biological activities and lower antigenicity have been highlighted[1].

RATIONALE AND OBJECTIVES

To evaluate the effect of temperature, pH and protease inhibitors on the proteolytic activity of *E. faecalis* FT132 on milk proteins, as well as to search for virulence genes and antibiotic resistance.

METHODS

E. faecalis FT132 was isolated from Brazilian raw bovine milk, and identified by 16S rDNA sequencing[2]. The proteolytic activity was initially detected in skim milk by cultivation at 37 °C / 24 h followed by tris-glycine SDS-PAGE . Caseins and whey protein hydrolysis were recorded at 3, 6, 9 and 24 h at 42 °C, and the influence of temperature, pH and protease inhibitors was also evaluated (Figure 1)[3].

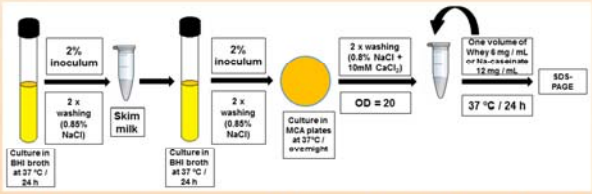

Figure 1. Procedures used to evaluate the proteolytic activity of *E. faecalis* FT132 with sodium caseinate and whey proteins in different temperatures, pH and in the presence of protease inhibitors.

Polymerase chain reaction (PCR) was used to detect virulence genes (*as*, *ace*, *cylA*, *efaA*, esp and *gelE*) and antibiotic resistance was evaluated by Kirby-Bauer disk diffusion susceptibility test.

RESULTS

Proteolytic activity of the strain in skim milk after incubation at 37 °C / 24 h is presented in Figure 2. Hydrolysis of caseins and whey proteins by *E. faecalis* FT132 was detected in the range of 30 °C to 42 °C / 24 h with higher activity observed at 42 °C (Fig. 3), while the optimal pH for proteolysis was 6.5 (Fig. 4). Caseins and whey proteins hydrolysis started after 3 h (Fig. 5) and were inhibited by EDTA (Fig. 6), suggesting a role for metalloproteases in the observed hydrolytic activity.

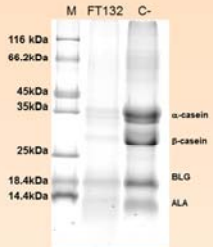

Figure 2. Proteolytic activity of *E. faecalis* FT132 in skim milk after incubation at 37 °C / h. M, molecular weicht marker; C-, negative control (skim milk).

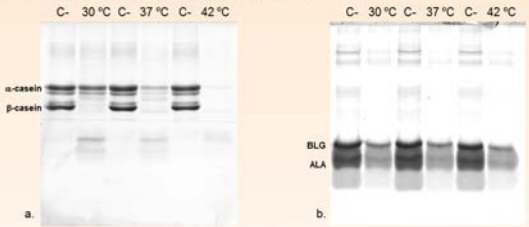

Figure 3. Proteolytic activity of *E. faecalis* FT132 in sodium caseinate (a) and whey (b) at 30, 37 and 42 °C. C-, negative control (sodium caseinate or whey).

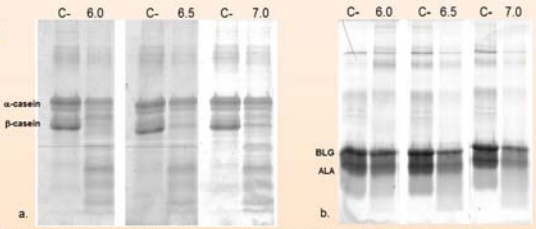

Figure 4. Proteolytic activity of *E. faecalis* FT132 in sodium caseinate (a) and whey (b) at 42 °C and pH 6.0, 6.5 and 7.0. C-, negative control (sodium caseinate).

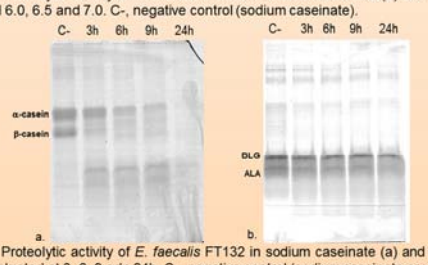

Figure 5. Proteolytic activity of *E. faecalis* FT132 in sodium caseinate (a) and whey (b) at 42 °C and evaluated at 3, 6, 9 adn 24h. C-, negative control (sodium caseinate or whey).

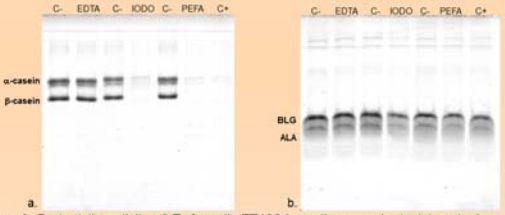

Figure 6. Proteolytic activity of *E. faecalis* FT132 in sodium caseinate (a) and whey (b) at 42 °C and evaluated in the presence of protease inhibitors. C-, negative control (sodium caseinate or whey).

Three virulence genes were detected in *E. faecalis* FT132 (*as*, *ace* and *gelE*) as presented in Table 1 and it was sensitive to ampicillin, ciprofloxacin, chloramphenicol, penicillin, rifampicin and vancomycin, but resistant to erythromycin and tetracycline (Table 2).

Table 1. Virulence genes detected in *E. faecalis* FT132 by PCR. (+), virulence gene was detected; (-), virulence gene was not detected.

	as	*ace*	*cylA*	*efaA*	*esp*	*gelE*
E. faecalis FT132	+	+	-	-	-	+

Table 2. Antibiotic resistance in *E. faecalis* FT132 determined by Kirby-Bauer disk diffusion susceptibility test.

	Ampicillin 10µg	Ciprofloxacin 5µg	Chloramphenicol 30µg	Erythromycin 15µg	Penicillin G 10U	Rifampicin 5µg	Tetracycline 30µg	Vancomicyn 30µg
Halo (cm)	27	21	13	0	21	21	0	18
Result	Susceptible	Susceptible	Intermediate	Resistant	Susceptible	Susceptible	Resistant	Susceptible

CONCLUSIONS

E. faecalis FT132 presented optimal proteolytic activity at 42 °C and pH 6.5. In addition, only a few virulence genes were found in this strain and these genes are widely distributed among food isolates. It also presented a narrow antibiotic resistance profile, indicating its potential to be applied for reduction of milk protein antigenicity.

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Evaluation of the probiotic potential of bacteriocinogenic and proteolytic lactic acid bacteria isolated from Brazilian raw cow's milk and cheese

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Key-words: Bacteriocin, Caco-2 cell, lactic acid bacteria, probiotic.

INTRODUCTION

Lactic acid bacteria (LAB) may present technological properties due to proteolytic activity and acid production, and their antimicrobial activity is of interest for application to food biopreservation. Some of these LAB also have potential to be used as probiotics, which are defined as "live microorganisms which provides a benefit to the host when provided in adequate quantities"(1).

OBJECTIVES

In this study, eight bacteriocinogenic and/or proteolytic LAB isolated from Brazilian raw cow's milk and cheese were identified by phenotypic and genotypic methods, and evaluated for some probiotic traits.

MATERIALS AND METHODS

Eight LAB strains, isolated from raw cow's milk and cheese, showing antimicrobial and/or proteolytic activity were selected for this study. The identification at genus level was done by biochemical tests: (i) Gram stain, (ii) catalase, (iv) heterofermentation from glucose, (v) growth in MRS with 20, 40 or 60g/l NaCl, (vi) growth in MRS at 10, 15 and 45ºC, and (vii) growth in MRS adjusted to pH9.6(2). LAB species were determined by 16S rDNA sequencing, using the primers 27F(3) and 1492R(4). The spot-on-the-lawn assay was used to determine the inhibitory spectrum of each LAB against *Listeria monocytogenes* and other bacteria. The strains were evaluated for resistance in acidified MRS broth (pH 2.0, 2.5 and 3.5), MRS broth with bile salts (Oxgall 0.3% w/v) and *in vitro* gastric conditions (NaCl 0.85% w/v solution with pepsin 3mg/ml, pH 2.0), at 37ºC, for 90 and 180 minutes. Viable cells population were enumerated on MRS agar with incubation at 25ºC/48h under anareobiosis. Adhesion to Caco-2 cells was evaluated after 30 minutes by plate count method and also, the adhered bacteria were hybridized with probe EUB 338, specific for the domain Bacteria, by FISH (fluorescence *in situ* hybridization) to be visualized with fluorescence microscope.

RESULTS

The LAB used in this work were identified by phenotypic tests as belonging to the genus *Enterococcus* (n=3), *Lactococcus* (n=4) and *Lactobacillus* (n=1). LAB species were determined by 16S rDNA sequencing, as shown in Table 1, as *Enterococus faecalis* (n=2), *Lactobacillus plantarum* (n=1) and *Streptococcus uberis* (n=5).

Table 1. Phenotypic and genotypic identification of LAB (Gram-positive, catalase negative) used in this study. N.D.= not determined.

Strain	Shape	Heterofermentation	Phenotypic characteristic	Growth in NaCl (g/l)			Growth at (ºC)			Growth in MRS, pH9.6	Phenotypic identification	Genotypic identification (16S rDNA)
				20	40	60	10	15	45			
FT86	Cocci	-	Bacteriocinogenic	+	+	-	+	N.D.	+	+	*Enterococcus*	*Streptococcus uberis*
FT126	Cocci	-	Bacteriocinogenic	+	-	-	+	N.D.	-	+	*Lactococcus*	*Streptococcus uberis*
FT132	Cocci	-	Proteolytic	+	+	+	+	N.D.	+	N.D.	*Enterococcus*	*Enterococcus faecalis*
FT133	Cocci	-	Proteolytic	+	+	+	+	N.D.	+	N.D.	*Enterococcus*	*Enterococcus faecalis*
FT190	Cocci	-	Bacteriocinogenic	+	+	-	-	N.D.	-	+	*Lactococcus*	*Streptococcus uberis*
FT191	Cocci	-	Bacteriocinogenic	+	-	-	-	N.D.	-	+	*Lactococcus*	*Streptococcus uberis*
FT192	Cocci	-	Bacteriocinogenic	+	+	-	+	N.D.	-	+	*Lactococcus*	*Streptococcus uberis*
FT259	Rod	-	Bacteriocinogenic / Proteolytic	+	+	+	-	+	-	N.D.	*Lactobacillus*	*Lactobacillus plantarum*

Among the eight LAB evaluated, six strains inhibited the growth of *Listeria monocytogenes* and other bacteria (Table 2).

Table 2. Inhibition spectra of LAB strains, using *Listeria monocytogenes* and other bacteria. Data show the diammeter of inhibition halos, in mm.

Indicator organism	LAB strains – diameter of inhibition halo (mm)					
	S. uberis FT86	*S. uberis* FT126	*S. uberis* FT190	*S. uberis* FT191	*S. uberis* FT192	*L. plantarum* FT259
Carnobacterium maltaromaticum A9b-	17	14	13	13	13	-
Cronobacter sakazakii ATCC 29004	-	-	-	-	-	-
Enterobacter aerogenes CDC 1535	-	-	-	-	-	-
Escherichia coli CDC 02A.2B	-	-	-	-	-	-
Klebsiella pneumoniae ATCC 10031	-	-	-	-	-	-
Lactobacillus sakei ATCC 15521	15	9	10	10	9	8
Leuconostoc mesenteroides A13	10	-	-	-	-	-
Listeria innocua ATCC 33090	22	20	25	27	25	13
Listeria monocytogenes IAL 633	23	12	20	20	19	12
Listeria monocytogenes ATCC 19115	26	-	30	30	28	17
Proteus mirabilis CDC 305	-	-	-	-	-	-
Pseudomonas aeruginosa ATCC 14502	-	-	-	-	-	-
Salmonella choleraesuis ATCC 13076	-	-	-	-	-	-
Staphylococcus aureus ATCC 29213	-	-	-	-	-	-
Staphylococcus epidermidis	-	-	-	-	-	-

Two proteolytic *Enterococcus faecalis* and one bacteriocinogenic and proteolytic *Lactobacillus plantarum* survived at pH 2.0, 2.5, 3.5, pepsin (pH2.0) and bile salts after 3h/37ºC (Figure 1). These strains showed 73.9±2.1 to 83.7±12.3% of adhesion to Caco-2 cells by plate count method and Figure 2 illustrated the bacterial adherence by the FISH assay.

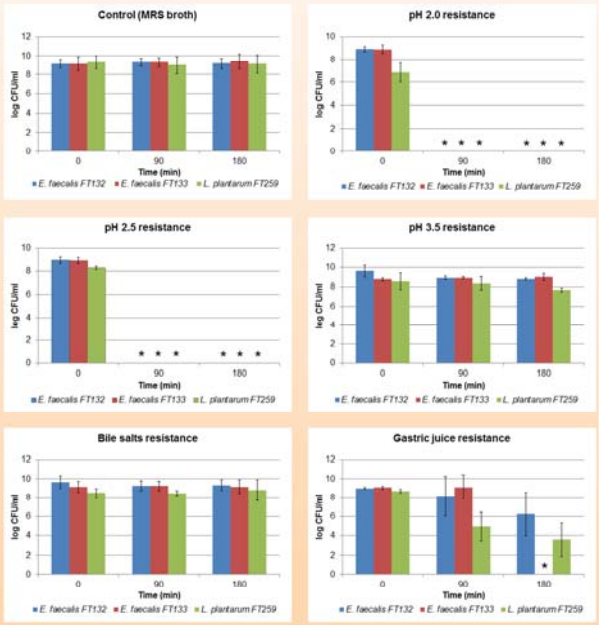

Figure 1. Quantification of LAB under acidic, gastric juice and bile salts conditions, in CFU/ml. * Population below quantification limit (2.4 log CFU/ml).

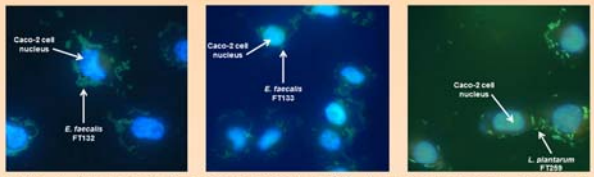

Figure 2. Adhesion of *Enterococcus faecalis* FT132, *Enterococcus faecalis* FT133 and *Lactobacillus plantarum* FT259 to Caco-2 cells, using probe EUB 338, by FISH (fluorescence *in situ* hybridization). The arrows indicate the Caco-2 cells and LAB.

CONCLUSIONS

- **LAB used in this work inhibited the growth of *L. monocytogenes*, with potential to biotechnological applications.**
- **Proteolytic activity of LAB tested may be a feature of interest for the dairy industry.**
- **LAB survived for 180 minutes at pH3.5, as well as to gastric conditions (bile salts and synthetic gastric juice). In addtion, FISH showed the adhesion of LAB to Caco-2 cell.**
- **In conclusion, two proteolytic *Enterococcus faecalis* isolated from milk, and one bacteriocinogenic and proteolytic *Lactobacillus plantarum* isolated from cheese, are potential probiotics candidates.**

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Identification of a Bacteriocinogenic Lactic Acid Bacteria Isolated From Raw Cow's Milk and Partial Characterization of its Antilisterial Bacteriocin

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INTRODUCTION

There is a demand for safe, healthy and minimally processed foods and this stimulates the search for new antimicrobial agents, such as the bacteriocins of lactic acid bacteria. Bacteriocins are peptides ribosomally synthesized by bacteria and can be degraded by digestive enzymes, representing a potentially safe alternative to classical food preservatives.

PURPOSE

To study a milk isolate bacteriocin-producing lactic acid bacterium (strain LAB 126) by phenotypical and molecular identification and partial physicochemical characterization of the antimicrobial peptide produced.

METHODS

1. LAB 126 isolation

Raw cow's milk → Serial dilution in bacteriological peptone 0.1% (w/v) → Isolation in MRS agar, 25ºC/72h, under anaerobiosis → Gram-positive and catalase negative isolates → Spot-on-the-lawn test for antilisterial activity → LAB 126 selected for further studies

2. LAB 126 phenotypic characterization[1]

LAB 126 → Gas production from glucose at 25ºC/48h; Growth in regular MRS broth at 10ºC (7 days) and 45ºC (48h); Growth in regular MRS broth in 2, 4 and 6.5% NaCl, at 25ºC/48h

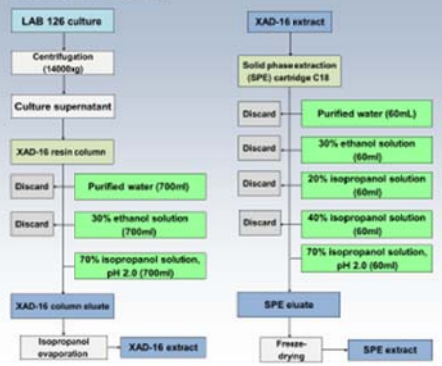
3. LAB 126 genotypic characterization

LAB 126 → Genomic DNA extraction and purification → PCR with primers 1492R and 27F (16S rDNA) → Amplicon purification → DNA sequencing → BLAST

Table 1. DNA sequence of the primers used in this study

Primer	Sequence (5' - 3')
27F[(2)]	AGAGTTTGATCMTGGCTCAG
1492R[(3)]	GCTTACCTTGTTACGACTT

4. Bacteriocin purification[(4)]



5. Bacteriocin analysis

Extracts containing bacteriocin were analysed by tricine SDS-PAGE and antimicrobial activity in extracts was measured by critical dilution assay using *L. monocytogenes* as indicator strain and expressed as arbitrary units per ml (AU/ml).

6. Antimicrobial spectrum

The growth inhibition of *Listeria* spp. and other bacteria were evaluated by the spot-on-the-lawn test.

RESULTS

LAB 126 was identified as *Streptococcus uberis* FT126 by phenotypic and genotypic tests (Figure 1 and Table 2), and it produced an inhibitory compound susceptible to degradation by proteases, indicating it was a bacteriocin (Figure 2).

Table 2. Phenotypic characterization of *Streptococcus uberis* FT126

Strain	Shape	Gas from glucose	Growth in NaCl (g/l)			Growth at (ºC)		Growth in MRS, pH9.6
			20	40	60	10	45	
FT126	Cocci	-	+	-	-	+	-	+

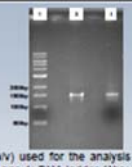


Figure 1. Agarose gel (1% w/v) used for the analysis of 16S rDNA amplicons from *Streptococcus uberis* FT126. Lane 1: DNA ladder 1kbp; lane 3: 16S rDNA 1500bp amplicon; lane 5: 16S rDNA 1500bp purified amplicon.

Figure 2. Protease susceptibility of the antilisterial compound produced by *Streptococcus uberis* FT126.

Streptococcus uberis FT126 inhibited the growth of *L. monocytogenes*, *Listeria innocua* and other lactic acid bacteria tested (Table 3).

Table 3. Antimicrobial spectrum of *Streptococcus uberis* FT126

Indicator organism	Diameter of inhibition halo (mm)
Carnobacterium maltaromaticum A9b-	14
Cronobacter sakazakii ATCC 29004	-
Enterobacter aerogenes CDC 1535	-
Escherichia coli CDC 02A 2B	-
Klebsiella pneumoniae ATCC 10031	-
Lactobacillus sakei ATCC 15521	9
Leuconostoc mesenteroides A13	-
Listeria innocua ATCC 33090	20
Listeria monocytogenes IAL 633	12
Listeria monocytogenes ATCC 19115	-
Proteus mirabilis CDC 305	-
Pseudomonas aeruginosa ATCC 14502	-
Salmonella choleraesuis ATCC 13076	-
Staphylococcus aureus ATCC 29213	-
Staphylococcus epidermidis ATCC 14990	-

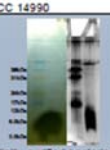


Figure 3. SDS-PAGE of the partially purified bacteriocin produced by *Streptococcus uberis* FT126. On the left, gel revealed with *L. monocytogenes*; on the right, gel revealed by silver staining.

Crude extract from BHI culture of *S. uberis* FT126 presented an activity of 6,400 AU/ml against *L. monocytogenes*. The yield of the partial purification carried out with XAD-SPE was calculated as 27%, by comparing total activities from crude and final extracts. Results from SDS-PAGE assays showed the molecular weight of the bacteriocin is in the range of 3.5 to 8.5kDa (Figure 3).

SIGNIFICANCE

The isolation of bacteriocin-producing LAB and the characterization of novel bacteriocins can contribute for improving food safety by providing new alternatives for food biopreservation approaches.

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