LUANA MARTINS PERIN

DIVERSIDADE MOLECULAR DA MICROBIOTA LÁTICA BACTERIOCINOGÊNICA DE LEITE DE CABRA E CARACTERIZAÇÃO DE SEU POTENCIAL BIOCONSERVADOR PARA A PRODUÇÃO DE QUEIJO MINAS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Medicina Veterinária, para obtenção do título de *Doctor Scientiae.*

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APROVADA: 05 de dezembro de 2014.

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“Talvez não tenha conseguido fazer o melhor, mas lutei para que o melhor fosse feito.

Não sou o que deveria ser, mas não sou o que era antes”.

(Martin Luther King Jr.)

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RESUMO

PERIN, Luana Martins, D.Sc., Universidade Federal de Viçosa, Dezembro de 2014. **Diversidade molecular da microbiota lática bacteriocinogênica de leite de cabra e caracterização de seu potencial bioconservador para a produção de queijo Minas.** Orientador: Luís Augusto Nero.

O leite de cabra cru é uma importante fonte de novos isolados de bactérias ácido láticas (BAL) bacteriocinogênicos. A constante demanda de consumidores por alimentos sem aditivos químicos justifica o estudo de alternativas para sua bioconservação. Este trabalho teve como objetivos isolar e identificar BAL bacteriocinogênicos presentes na microbiota autóctone do leite de cabra cru, caracterizar suas bacteriocinas produzidas, seu potencial de virulência e avaliar seu potencial bioconservador na produção de queijos Minas produzido com leite de cabra cru. Como aspecto adicional de segurança, aminas biogênicas (AB) presentes nos queijos produzidos foram quantificadas. BAL foram isoladas do leite de cabra cru usando meios de cultura seletivos e submetidas à avaliação de seu potencial bacteriocinogênico a partir de testes moleculares e fenotípicos usando como indicador *Listeria monocytogenes* ATCC 7644. O gene codificador de nisina dos isolados positivos foi submetido a sequenciamento para identificação de possíveis variações em sua composição de aminoácidos. Isolados caracterizados como bacteriocinogênicos foram identificados a partir do sequenciamento dos genes 16S rRNA e adicionalmente do gene *pheS* para identificação das espécies de *Enterococcus*. Esses isolados foram agrupados de acordo com sua similaridade genética por rep-PCR e a partir dos perfis gerados, alguns isolados foram selecionados e submetidos a testes fenotípicos e moleculares para identificação de seu potencial de virulência. O isolado *Lactococcus lactis* subsp. *lactis* GLc05 foi escolhido pelo seu interessante potencial bacteriocinogênico e ausência de fatores de virulência para produção de queijo Minas utilizando leite de cabra cru. A microbiota dos queijos

com (A) e sem (B) adição de *L. lactis* subsp. *lactis* GLc05 foi analisada usando métodos cultura-dependentes (meios de cultura seletivos) e -independentes (rep-PCR e DGGE). A quantificação de AB nos queijos Minas foi realizada por HPLC. Produtores de bacteriocinas foram identificados como *Lactococcus* spp. (24) e *Enterococcus* spp. (33). Entre os isolados, 9 *Lactococcus lactis* foram identificados como produtores de uma nova variante de nisina ainda não descrita e com amplo espectro de ação. Testes de caracterização do potencial de virulência demonstraram a presença de genes relacionados a patogenicidade e expressão de alguns desses fatores em alguns isolados identificados como *Lactococcus* spp. Por outro lado, alguns isolados identificados como *Enterococcus* spp., usualmente considerados patógenos oportunistas, não apresentaram esses fatores. A análise da microbiota dos queijos A e B usando métodos cultura-dependentes e -independentes mostrou que *L. lactis* subsp. *lactis* GLc05 foi capaz de controlar a população de cocos coagulase-positivo e causar mudanças na microbiota autóctone em queijo Minas produzido com leite de cabra cru. Altas concentrações de AB foram encontradas em queijo Minas produzido com leite de cabra cru, revelando importância de garantir a qualidade higiênico-sanitária do leite utilizado, porém foram significativamente menores nos queijos Minas adicionados de *L. lactis* subsp. *lactis* GLc05. Os resultados indicam que este isolado pode ser utilizado para produção de queijos produzidos com leite cru, pois foi capaz de controlar populações de micro-organismos patogênicos e as concentrações de AB.

ABSTRACT

PERIN, Luana Martins, D.Sc., Universidade Federal de Viçosa, December, 2014. **Molecular diversity of bacteriocinogenic lactic microbiota from goat milk and characterization of its bioconservative potential for producing a Minas cheese.** Adviser: Luís Augusto Nero.

The raw goat milk is an important source of new bacteriocinogenic lactic acid bacteria (LAB) strains. The constant demand of consumers for foods without chemical additives justifies the study of alternatives for biopreservation. This study aimed to isolate and identify bacteriocinogenic LAB strains present in the autochthonous microbiota of raw goat milk, characterize their bacteriocins, virulence potential and evaluate its bioconservative potential in Minas cheese manufactured with raw goat milk. As an additional preservative aspect, biogenic amines (BA) present in the produced cheeses were quantified. LAB were isolated from raw goat milk using selective media and subjected to evaluation of their bacteriocinogenic potential from molecular and phenotypic tests using *Listeria monocytogenes* ATCC 7644 as target. Nisin positive strains were submitted to gene sequencing in order to identify possible variations in amino acid composition of the peptide codified. Strains characterized as bacteriocinogenic were identified by 16S rRNA with additional *pheS* sequencing to identify *Enterecoccus* species. These strains were grouped according to their genetic similarity by rep-PCR and some isolates were selected and submitted to further phenotypic and molecular tests for identification of their virulence potential. *Lactococcus lactis* subsp. *lactis* GLc05 was selected based on its interesting bacteriocinogenic potential and absence of virulence factors for producing Minas cheese manufactureted with raw goat milk. The cheeses microbiota with (A) and without (B) addition of GLc05 was analyzed using culture-dependent (selective media) and -independent (rep-PCR and DGGE) methods. The BA quantification in the Minas

cheeses was performed by HPLC. Bacteriocinogenic strains were identified as *Lactococcus* spp. (24) and *Enterococcus* spp. (33). Among them, 9 *Lactococcus lactis* were identified as producing a new variant of nisin with broad antimicrobial activity spectrum. Characterization of virulence potential showed the presence of genes related to pathogenicity and their expression in some isolates identified as *Lactococcus* spp. Moreover, some isolates identified as *Enterococcus* spp. usually considered opportunistic pathogens, did not presented these genes. Analysis of the cheeses A and B using culture-dependent and -independent methods showed that *L. lactis* subsp. *lactis* GLc05 was able to control the coagulase-positive cocci population and was capable to cause changes in the microbiota composition in Minas cheese manufactured with raw goat milk. High concentrations of BA were found in the cheeses, revealing the importance of ensuring the sanitary quality of the milk used. However BA concentrations were significantly lower in cheeses A. The results indicate that *L. lactis* subsp. *lactis* GLc05 can be used for the production of cheeses manufactured with raw milk as it is capable of controlling pathogenic microorganisms populations and also the concentrations of BA.

INTRODUÇÃO

A caprinocultura leiteira no Brasil é uma atividade que ainda se apresenta em fase de desenvolvimento. Incentivos do governo brasileiro e de instituições governamentais ainda são escassos e os produtores não recebem treinamento adequado para as práticas de produção de leite e derivados. Ainda assim, o volume de leite produzido e o número de produtores atuando na atividade vêm aumentando a cada ano, demonstrando um crescente interesse dos consumidores e da indústria no leite de cabra.

Outro entrave no crescimento da atividade é o fato que, cerca de 80% do leite de cabra que é produzido no Brasil é comercializado como leite fluido, gerando altos custos com transporte e inviabilizando sua venda para mercados distantes. Uma importante alternativa para comercialização do produto é a produção de derivados, principalmente queijos, realidade de muitos países da Europa. A produção de queijos diferenciados agrega valor ao produto e atinge um mercado consumidor refinado, capaz de pagar valor proporcional ao produto desenvolvido.

A microbiota autóctone do leite de cabra e algumas características específicas de seus componentes conferem a esses queijos características sensoriais típicas, como odor, sabor e textura. A correlação entre as características do produto e o micro-organismo causador da interferência pode ser estabelecida através de algumas técnicas moleculares cultura-independentes, como o DGGE (Eletroforese em Gel de Gradiente Desnaturante) e TGGE (Eletroforese em Gel de Gradiente de Temperatura). Essas técnicas fornecem importantes informações sobre a ecologia e a dinâmica das populações microbianas presentes no alimento. Esses dados são importantes ferramentas para o monitoramento adequado do processo de produção e maturação desses queijos.

Muitas das características típicas dos queijos de cabra são provenientes das transformações realizadas pelas Bactérias Ácido Láticas (BAL), que são capazes de

produzir diversas substâncias desejáveis para uso tecnológico. BAL ainda são capazes de produzir substâncias antimicrobianas, principalmente bacteriocinas, que inibem a multiplicação de bactérias patogênicas e deteriorantes presentes no alimento, sendo de particular interesse pelas indústrias para aplicação como bioconservantes em alimentos. A escolha das cepas de BAL para uso em alimentos deve ser realizada com cautela, com a caracterização de sua inocuidade. Caso a cepa de interesse possua propriedades virulentas, a solução seria a purificação das bacteriocinas produzidas para aplicação nos alimentos.

O uso de BAL bacteriocinogênicas em alimentos é uma alternativa natural para serem usados como co-adjuvantes ao uso de tratamentos térmicos e de substâncias químicas. Por essa razão é crescente a demanda por pesquisas para descobrir novos isolados interessantes para serem aplicadas em alimentos, assim como o estudo de sua interferência na microbiota autóctone da matriz alimentar onde serão aplicadas.

REVISÃO BIBLIOGRÁFICA

1. Caprinocultura leiteira no contexto mundial e brasileiro e o leite de cabra

A caprinocultura leiteira é a segunda atividade pecuária que mais se desenvolve em todo o mundo, apresentando um crescimento de aproximadamente 33% entre os anos de 2002 e 2012. Em 2012 a produção mundial de leite de cabra foi de 17 milhões de toneladas, sendo menor do que as quantidades produzidas de leite de vaca e de búfala (FAOSTAT, 2014).

No Brasil essa atividade é ainda recente, o que pode justificar sua baixa produção em níveis mundiais, principalmente quando comparado com alguns países da Ásia, África e Europa, onde é desenvolvida como uma das principais fontes de renda de produtores rurais e indústrias, e já possui mercado consumidor bem definido e estável. Ainda assim, o Brasil é o maior produtor de leite de cabra da América Latina, apresentando produção de 150 mil toneladas no ano de 2012 (FAOSTAT, 2014).

A região Nordeste do Brasil possui 90% da população nacional de caprinos concentrada principalmente na Bahia, Pernambuco e Piauí. Apesar disso, as regiões Sul e Sudeste por apresentarem uma cadeia produtiva mais organizada e estruturada, contribuem com 55% da produção nacional de leite de cabra. O leite pasteurizado, congelado, UHT e em pó são as principais formas de consumo do leite de cabra no Brasil. Até o momento, a sua exploração tecnológica para produção de outros produtos ainda é limitada e, menos de 1% do total de leite que é produzido, é destinado à produção de derivados, como queijos, iogurtes, doces, sorvetes e cosméticos.

A industrialização do leite e a produção de derivados requerem legislações específicas, dessa forma a publicação da Instrução Normativa 37, em 2000 (Brasil, 2000) foi um importante reflexo do desenvolvimento dessa atividade no Brasil. A IN 37

teve o objetivo de regulamentar as condições de produção, a identidade e os requisitos mínimos de qualidade do leite de cabra destinado ao consumo humano. Assegurando uma produção de alta qualidade e em condições higiênico-sanitárias adequadas, estimulando o aumento do consumo de leite de cabra e subprodutos.

O leite de cabra possui algumas características que o diferencia e determinam vantagens nutricionais e terapêuticas quando comparados com o leite de vaca:

- a caseína não possui a fração α-caseína, sendo majoritariamente constituído de β-caseína, não induzindo alergias alimentares ou outros problemas no trato digestivo, acometimentos comuns em crianças durante os três primeiros anos de vida (Haenlein, 2004; Park, 2007);
- não possui β-caroteno, possuindo coloração branca;
- tem sabor forte, devido à liberação de ácidos graxos de cadeia curta durante sua manipulação;
- suas proteínas são mais rapidamente digeridas e os aminoácidos são absorvidos com maior eficiência do que aminoácidos do leite de vaca (Jenness, 1980; Jandal, 1996);
- os glóbulos de gordura são menores e possuem maior superfície de contato, possibilitando que as lipases presentes no trato digestivo degradem os lipídeos mais rapidamente, e facilitando a digestão da gordura do leite (Jenness, 1980; Jandal, 1996);
- possui maior quantidade de triglicerídeos de cadeia média (MCT), o que diminui a síntese de colesterol endógeno, reduzindo os níveis de colesterol total e da fração LDL (Haenlein, 2004);
- possui natureza alcalina, devido à maior quantidade de proteína e a um arranjo diferente de fosfatos, sendo vantagem para pessoas com problemas de acidez estomacal (Saini & Gill, 1991).

Independentemente da espécie animal, a microbiota autóctone do leite possui contagens bacterianas muito maiores do que as contagens de fungos. Mais de 100 gêneros e 400 espécies microbianas já foram encontradas, sendo em sua maioria bactérias Gram-negativas, e Gram-positivas catalase positivas; seguidos por leveduras, BAL e fungos (Montel et al., 2014). BAL são historicamente foco da maioria dos estudos e muitas vezes são consideradas como principal constituinte do leite cru (Bonetta et al., 2008; Dolci, 2009; Dolci et al., 2010; Quigley et al., 2011).

2. Bactérias ácido láticas (BAL)

Até o momento cerca de 400 espécies de BAL são reconhecidas, e geralmente são classificadas como pertencentes a quatro famílias e sete gêneros. De acordo com Zhang et al. (2011), são classificadas como: família Lactobacillaeae (gêneros *Lactobacillus* e *Pediococcus*), família Leuconostocaceae (gêneros *Oenococcus* e *Leuconostoc*), família Enterococcoceae (gênero *Enterococcus*) e família Streptococcaceae (gêneros *Lactococcus* e *Streptococcus*).

O termo BAL refere-se principalmente ao seu metabolismo basal, que a partir da fermentação de açúcares produzem, principalmente, ácido lático. Devido à rápida produção de ácido lático, são industrialmente importantes e usadas em todo o mundo objetivando a produção de alimentos fermentados, como queijos. Além disso, são capazes de produzir outras substâncias desejáveis que contribuem para características sensoriais típicas do produto final, modificando o sabor, textura e odor.

Outro aspecto benéfico de BAL é a sua capacidade de auxiliar na garantia de inocuidade alimentar. Muitos estudos mostram que BAL desempenham atividade inibitória considerável sobre micro-organismos patogênicos e deteriorantes presentes nos alimentos (Nero et al., 2008; Dal Bello et al., 2010; Ortolani et al., 2010b; Moraes et

al., 2012; Perin et al., 2012). A interferência de BAL sobre esses organismos pode ocorrer de várias formas: competição por oxigênio, competição por sítios de ligação e produção de substâncias antagonistas (de Martinis et al., 2002). Dentre as substâncias produzidas por BAL com potencial antimicrobiano, se destacam peróxido de hidrogênio, diacetil, dióxido de carbono, peptídeos antifúngicos, substâncias antimicrobianas de baixa massa molar (reuterina, reutericiclina e ácido piroglutâmico) e principalmente bacteriocinas (Leroy & De Vuyst, 2004; Cotter et al., 2005; Chen & Hoover, 2006).

2.1. Bacteriocinas

A primeira descrição da inibição mediada por bacteriocinas foi descrita há cerca de 80 anos, quando o antagonismo entre cepas de *Escherichia coli* foi descoberto. As substâncias antimicrobianas produzidas por *E. coli* foram originalmente nomeadas como colicinas, como referência ao organismo produtor. Atualmente, essas substâncias recebem a denominação de bacteriocinas. Durante muitos anos o estudo de bacteriocinas foi concentrado em bactérias Gram-negativas, o que permitiu o desenvolvimento de técnicas de identificação e caracterização que são utilizadas até hoje (Cotter et al., 2005).

Bacteriocinas são pequenos peptídeos termo-resistentes de síntese ribossomal, biologicamente ativos que variam no seu espectro e modo de ação, massa molecular, origem genética, e propriedades bioquímicas (Abee et al., 1995). A Tabela 1 mostra a classificação das bacteriocinas sugerida por Cotter et al. (2005).

Tabela 1. Classificação das bacteriocinas de acordo com Cotter et al. (2005).

Classificação	Características	Modo de ação sobre o micro-organismo alvo	Exemplos
Classe I	Possuem resíduos de lantionina ou β-metilantionina		
Ia	São alongados, flexíveis e carregados positivamente	Formação de poros na membrana citoplasmática	Nisina, lacticina 481, subtilina
Ib	São globulares, rígidos e neutros	Interferência nas reações enzimáticas essenciais	Lacticina 3147, citolisina
Classe II	São pequenos (<10 KDa), termoestáveis e helicoidais		
IIa	Semelhantes a pediocina PA-1, com região conservada pediocina-box	Formação de poros na membrana citoplasmática	Enterocina P, A e B, leucocina A, sakacina, pediocina PA-1
IIb	Dois-componentes, sintetizadas sem peptídeo líder, requerem complexo sistema de transporte	Formação de poros na membrana citoplasmática	Lactococina G, lactacina F, enterocina L50, plantaricina
Classe III	Bacteriolisinas, grandes e termo-lábeis	Causam hidrólise da membrana citoplasmática	Enterocina AS-48

A formação de poros pelas bactérias na membrana citoplasmática do micro-organismo alvo não é um mecanismo totalmente elucidado, mas basicamente ocorre a ligação da bacteriocina ao lipídeo II (principal transportador das subunidades dos peptideoglicanos do citoplasma para a parede celular) impedindo a síntese correta da parede celular, levando à morte celular. Outra teoria é que a bacteriocina pode utilizar o lipídio II como uma molécula de acoplamento para iniciar o processo de inserção da membrana e formação de poros, levando à morte celular (Cotter et al., 2005).

Diversas espécies de bactérias Gram-positivas e Gram-negativas são capazes de produzir bacteriocinas. Entretanto, BAL, por serem organismos que possuem o status GRAS (Geralmente Reconhecidas como Seguras), possuem particular interesse por parte das indústrias para o uso em alimentos (Deegan et al., 2006), funcionando como uma barreira bactericida ajudando a reduzir a susceptibilidade dos alimentos à multiplicação de micro-organismos patogênicos e deteriorantes (de Martinis et al., 2002; Ortolani et al., 2010a; Ortolani et al., 2010b; Settanni et al., 2011; Pingitore et al., 2012).

As bacteriocinas produzidas por BAL podem possuir amplo ou limitado espectro de ação, mas geralmente possuem ação contra bactérias Gram-positivas, que são intimamente relacionadas ao micro-organismo produtor, e também são capazes de inibir patógenos como *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium perfringens* e *Bacillus cereus* (de Martinis et al., 2002; Castellano et al., 2008). Evidentemente, o micro-organismo produtor possui mecanismos de imunidade contra a sua bacteriocina produzida (Klaenhammer, 1988; De Vuyst & Leroy, 2007).

Bacteriocinas possuem ação restrita contra bactérias Gram-negativas, devido à presença da membrana externa (Rodríguez et al., 2000; Deegan et al., 2006; Ortolani et al., 2010a). No entanto, as bacteriocinas podem ser usadas em combinação com outros tratamentos que afetem a integridade da membrana de bactérias Gram-negativas (Cotter et al., 2005). Acuña et al. (2012) descreveram o desenvolvimento e a expressão de um novo peptídeo híbrido, combinando enterocina CRL35 e microcina V. O novo peptídeo foi capaz de inibir o desenvolvimento de bactérias Gram-negativas, como isolados clínicos de *Escherichia coli* enterohemorrágica, além de possuir ação contra *L. monocytogenes*, e outras bactérias patogênicas.

O sucesso do uso de bioconservantes estimula o surgimento de estudos objetivando a caracterização e a descoberta de novas bacteriocinas para serem aplicadas

em diversos alimentos (D'Angelis et al., 2009; Ortolani et al., 2010a; Borrero et al., 2011; Todorov et al., 2011; Moraes et al., 2012; Perin et al., 2012). As bacteriocinas podem ser incorporadas nos alimentos em sua forma purificada ou semi-purificada, ou a BAL produtora pode ser incorporada diretamente no alimento já fermentado, ou substituindo totalmente ou parcialmente a cultura starter em alimentos fermentados (Deegan et al., 2006).

A aplicação direta da BAL bacteriocinogênica nos alimentos, muitas vezes, pode ser mais atrativa para as indústrias, pois não necessita de aprovação legal para ser incorporada. Por outro lado, alguns elementos da matriz alimentar e os processamentos que serão submetidos podem interferir com a produção de bacteriocinas pela BAL (Deegan et al., 2006). É importante salientar que BAL produtoras de bacteriocinas devem preferencialmente ser aplicadas nos mesmos alimentos dos quais foram isoladas, pois são mais adaptadas a estas matrizes e serão mais competitivas em relação às BAL de outras origens (Bromberg et al., 2005).

2.2. Lantibióticos e nisina

Lantibióticos possuem elevada proporção de aminoácidos incomuns em sua estrutura, incluindo os aminoácidos do grupo tioéter lantionina (Lan) e metil lantionina (MeLan) e ainda uma série de aminoácidos modificados, como, 2,3-dihidroalanina (Dha) e 2,3-dihidrobutirina (Dhb). A lantionina é formada quando a ligação dupla de Dha reage com o tiol de um resíduo de cisteína vizinho. Já a metil lantionina é formada quando essa reação ocorre com Dhb. Como consequência destas pontes intramoleculares, lantibióticos são estruturas policíclicas contidas de vários anéis de lantionina. Esses anéis protegem os lantibióticos contra degradação por proteases, ao tratamento térmico e ajudam na estabilidade da molécula (McAuliffe et al., 2001;

Alkhatib et al., 2012).

Os genes dos lantibióticos são dispostos em operons que são induzidos por seus próprios produtos ou por fatores ambientais. Segundo de Vos et al. (1995), a nomenclatura genérica para os lantibióticos inclui o peptídeo precursor (LanA), as enzimas responsáveis pela especificidade das reações de modificação (LanB, C/LanM), proteínas acessórias, incluindo as proteases de processamento responsáveis pela remoção do peptídeo líder (LanP), a superfamília-ABC, proteínas de transporte envolvidas na translocação de peptídeos (LanT), proteínas reguladoras (LanR, K), proteínas responsáveis pelos mecanismos de imunidade (LanI, F, E e G), e outros genes ainda sem homólogos no banco de dados (McAuliffe et al., 2001).

O lantibiótico nisina é um polipeptídeo pequeno (3,4 KDa), composto de 34 aminoácidos. Os genes relacionados a produção de nisina estão dispostos no operon *nis*ABCEFGKIPRT e são encontrados em transposons. O gene estrutural *lan*A, encontrado em todos os lantibióticos (*nis*A no caso da nisina), codifica a síntese ribossomal de peptídeos precursores conhecidos como "pré-peptídeos". Ao contrário dos peptídeos maduros, estes pré-lantibióticos são biologicamente inativos e possuem a região N-terminal, ou peptídeo líder, ligado ao pré-peptídeo na região C-terminal. Dessa forma, NisA corresponde a um peptídeo precursor de 57 aminoácidos, 23 dos quais formam a sequência líder que é clivada do peptídeo maduro na última etapa da biossíntese da nisina e 34 aminoácidos que correspondem o peptídeo ativo. A desidratase NisB, catalisa a desidratação de serina e treonina no pré-peptídeo através do reconhecimento específico das partes da sequência líder. Na etapa de maturação, a pré-nisina desidratada é modificada pela ciclase NisC, que catalisa a formação dos anéis de lantionina e metil lantionina. O sistema de transporte ABC (NisT) exporta a pré-nisina modificada, que se torna ativa apenas quando a sequência sinal é clivada pela protease de membrana NisP (Alkhatib et al., 2012).

A nisina é produzida por *Lactococcus lactis* e foi descoberta em 1928 sendo a primeira bacteriocina a ser comercializada, na Inglaterra em 1953, e desde então foi aprovada para uso em mais de 48 países. Em 1969 foi considerada pela Food and Agriculture Organization (FAO) como segura para ser usada em alimentos e em 1988, foi aprovada pelo Food and Drug Agency (FDA) para ser utilizada em queijos pasteurizados (Cotter et al., 2005).

No Brasil, até o momento, somente a nisina é permitida legalmente para ser utilizada como aditivo (Nisaplin™ é uma das formas comerciais, e distribuída pela DuPont, EUA). O seu uso é regulamentado pela Agência Nacional de Vigilância Sanitária (ANVISA) que permite que o aditivo intencional nisina possa ser utilizado no limite máximo de 12,5 mg/kg, como conservador na tecnologia de fabricação de queijo pasteurizado, queijo fundido e requeijão (ANVISA, 1996).

A nisina possui ação inibitória contra outros micro-organismos Gram-positivos, como *Lactococcus* spp., *Streptococcus* spp., *Staphylococcus* spp. e *Listeria* spp., além de prevenir o desenvolvimento de esporos de *Bacillus* spp. e *Clostridium* spp. A nisina utiliza o lipídio II como uma molécula de acoplamento, possuindo ação bactericida dupla, devido a inibição da biossíntese de peptideoglicanos e da formação de poros na membrana citoplasmática do micro-organismo alvo (Field et al., 2008).

A nisina A foi a primeira variante a ser descoberta em 1928 (Rogers & Whittier, 1928), e cinco outras variantes naturais de nisina já foram descritos. A nisina Z, F e Q também são produzidas por *L. lactis*, e a nisina U e U2 são produzidas por *Streptococcus* sp. As estruturas e as sequências de aminoácidos das variantes de nisina estão apresentadas na Figura 1.

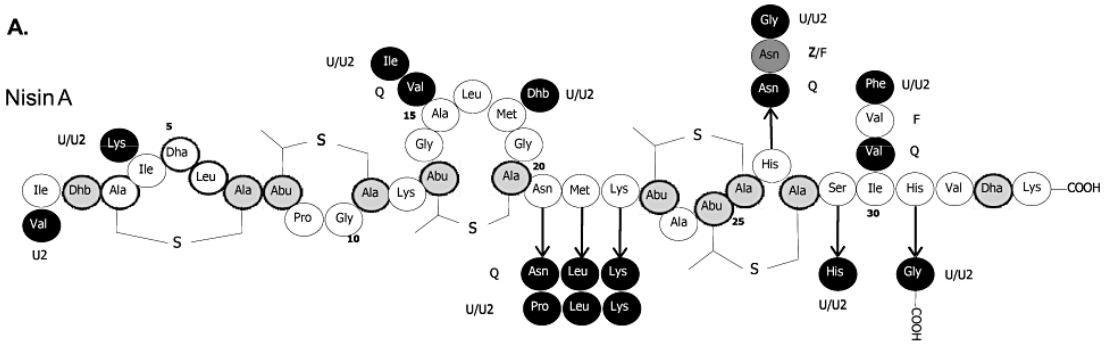

Figura 1. Estrutura da variante natural nisina A e estruturas putativas das variantes nisina Z, Q, U e U2. Círculos pretos indicam diferenças de aminoácidos entre as variantes de nisina (Piper et al., 2011)

A nisina Z foi purificada pela primeira vez por Mulders et al. (1991) a partir da cepa de *L. lactis* NIZO 22186 isolada de produtos lácteos e se difere da nisina A por apenas um aminoácido (Histidina substituída por Asparagina na posição 27). A nisina F produzida pela cepa de *L. lactis* F10 (De Kwaadsteniet et al., 2008) se difere da nisina A por dois aminoácidos (Histidina substituída por Asparagina na posição 27, igual a nisina Z; e Isoleucina substituída por Valina na posição 30). A nisina Q produzida pelos isolados de *L. lactis* 61 e 14 (Fukao et al., 2008), possui as mesmas duas variações apresentadas pela nisina F e outras duas variações adicionais (Alanina substituída por Valina na posição 15; e Metionina substituída por Leucina na posição 21). A nisina U produzida por *S. uberis* 42 e nisina U2 produzida por *S. agalactiae* D536 se diferem da nisina A por 9 e 10 aminoácidos respectivamente (Wirawan et al., 2006). Enquanto as outras variantes de nisina são constituídas por 34 aminoácidos, esses peptídeos são constituídos de 31 aminoácidos (Rollema et al., 1996). Apesar de possuírem diferenças nas sequências dos aminoácidos, as variantes possuem atividade antimicrobiana muito similar.

2.3. Fatores de virulência

A seleção da cepa de BAL para ser aplicada em alimentos deve ser muito criteriosa. Muitas BAL constituintes da microbiota autóctone do leite podem possuir diversos genes associados à virulência. Mesmo que muitas cepas não sejam capazes de expressá-los, não sendo potencialmente patogênicas, elas podem atuar como reservatórios e transferi-los para outros micro-organismos (Herreros et al., 2005; Barbosa et al., 2010).

Uma grande preocupação para a indústria de alimentos seria a utilização de cepas que possuam genes de resistência a antimicrobianos. BAL presentes no leite ou carne obtidos a partir de animais que foram tratados com antibióticos sofrem pressão seletiva adquirindo fenótipos de resistência que podem ser transferidos pela cadeia alimentar (Ammor et al., 2007; Toomey et al., 2010). Produtos que não sofrem tratamento térmico antes do consumo constituem importantes veículos de bactérias resistentes a antimicrobianos, com uma ligação direta entre a microbiota endógena dos animais e do trato digestivo humano. A ocorrência de resistência não é um fator de virulência por si só, mas infecções por micro-organismos resistentes podem complicar a evolução de doenças aumentado o número de internações hospitalares e dobrando a morbidade e mortalidade (Levy & Marshall, 2004).

A resistência a antimicrobianos pode ser intrínseca ou adquirida (através de uma ou mais mutações, ou pela incorporação de novos genes). A ocorrência da resistência a antimicrobianos em comunidades microbianas é agravada pela transferência horizontal de genes por plasmídeos conjugativos, transposons, presença de integrons, elementos de inserção, bem como bacteriófagos líticos e temperados (Clementi & Aquilanti, 2011). A transferência de plasmídeos de resistência por

conjugação a partir de *Enterococcus* ou espécies de *Staphylococcus* para outras BAL já foi descrita (Ammor et al., 2007).

Normalmente BAL são resistentes a alguns antimicrobianos das famílias dos β-lactâmicos, cefalosporinas e aminoglicosídeos (Herreros et al., 2005). A maioria dos dados sobre resistência a antimicrobianos em BAL é focada em *Enterococcus* (Barbosa et al., 2010; Hadji-Sfaxi et al., 2011; Moraes et al., 2012), e pouco se sabe sobre a presença desses genes em cepas starters e o potencial de transferência dos genes para patógenos (Mathur & Singh, 2005). *Enterococcus* resistentes à vancomicina (VRE) surgiram na última década como uma causa frequente de infecções hospitalares (Mathur & Singh, 2005). Em *Enterococcus* já foram descritos seis tipos de resistência à vancomicina; dois deles, VanA e VanB, podem estar localizados em plasmídeos transferíveis (Courvalin, 2006).

Diferentes métodos podem ser utilizados na triagem da identificação da resistência aos antimicrobianos em bactérias, como o Etest (bioMérieux, Marcy l'Etoile, França) e o MICE (Oxoid Ltd., Basingstoke, Inglaterra) que permitem a identificação da concentração mínima inibitória (CIM) dos antibióticos, testes de difusão em discos, e utilização de meios de cultura. Cada um dos métodos tem suas limitações e muitas vezes podem ocorrer discordâncias entre os resultados. Variações de concentração do inóculo, temperatura e condições de incubação podem modificar os resultados obtidos. Ainda, BAL não se desenvolvem bem em meios de cultura usados em testes de resistência, como o Mueller Hinton (Difco) ou Isosensitest (Oxoid), e o ágar de Mann Rogosa & Sharpe (MRS), usualmente utilizado para recuperação e multiplicação de BAL, pode inativar alguns antibióticos. Dessa forma, é importante que os testes fenotípicos sejam complementados por testes moleculares para identificação da presença de genes de virulência ou sua expressão (Ammor et al., 2007).

No caso de alimentos fermentados, muitas BAL são capazes de converter aminoácidos em aminas biogênicas (AB) via descarboxilase ou desaminase durante o processo de fermentação. Por essa razão o catabolismo de aminoácidos por BAL pode afetar a qualidade e a segurança de alimentos fermentados (Coton et al., 2010). AB podem ocorrer em diversos tipos de alimentos, incluindo leite e derivados (Bover-Cid & Holzapfel, 1999), e em certos níveis (ingestão de 50-100 mg) constituem uma importante causa de intoxicação alimentar em consumidores susceptíveis. A produção destes compostos é atribuída principalmente a *Enterococcus* spp. (Giraffa et al., 1995).

Histamina e Tiramina são as AB mais estudadas devido aos efeitos toxicológicos derivados de suas propriedades vasoativas e psicoativas. Histamina é conhecida por ser o agente causador do envenenamento escombróide, associado ao consumo de alguns peixes (Bover-Cid & Holzapfel, 1999) e geralmente causa queda da pressão arterial, dor de cabeça, pruridos na pele, vômitos e diarreias. A doença geralmente se resolve em 3 horas, mas pode permanecer por alguns dias em casos mais graves. Já a tiramina é usualmente a AB mais frequente em alimentos fermentados e o seu consumo em altos níveis causa o típico fenômeno chamado “reação do queijo”. A tiramina é normalmente metabolizada pela enzima mono-amino oxidase (MAO) localizada no trato digestivo e no fígado. Em pacientes em tratamento com antidepressivos que utilizam fármacos inibidores da MAO (IMAO), a tiramina não é metabolizada causando enxaquecas alimentares e crises hipertensivas (Landete et al., 2007). As outras AB, como cadaverina, putrescina, diaminas podem atuar potencializando os efeitos da histamina e tiramina (Bover-Cid & Holzapfel, 1999; Rivas et al., 2005).

A ocorrência de AB em altos níveis em alimentos pode ser indicativa de processos de deterioração ou elaboração do produto em más condições higiênico-sanitárias. A detecção da produção das AB pode ser realizada de forma qualitativa,

através de ensaios fenotípicos e moleculares, com uso da PCR comum (Rivas et al., 2005; Landete et al., 2007; Coton et al., 2010) ou de forma quantitativa, com uso de técnicas como a Cromatografia Líquida de Alta Performance (HPLC).

Além da presença de genes de resistência a antibióticos e produção de AB, outros fatores de virulência também são importantes de serem pesquisados em BAL, como: produção de gelatinase, que causa hidrólise de colágenos, podendo estar envolvida no início e propagação de processos inflamatórios (Lopes et al., 2006); citolisinas, uma exotoxina de efeito hemolítico e bacteriocinogênica, capaz de invadir o sistema imune do hospedeiro e pode causar lise de eritrócitos (Franz & Holzapfel, 2004); substâncias de agregação, que permitem o contato entre as células para conjugação e consequente transmissão de plasmídeos de virulência e também tem importante papel na translocação de *Enterococcus* para dentro de células epiteliais (Hendrickx et al., 2009); proteínas de superfície, que são proteínas ancoradas a parede celular que participam na formação de biofilmes (Hendrickx et al., 2009); hialuronidase, que facilita a dispersão da bactéria e suas toxinas, causando danos e degradação dos tecidos do hospedeiro (Franz & Holzapfel, 2004); expressão de antígenos relacionados a endocardite em *E. faecalis*; dentre outros fatores.

O uso de métodos fenotípicos para identificação desses fatores, muitas vezes pode gerar resultados falso negativos, pois alguns elementos relacionados ao teste "*in vitro*" podem interferir na expressão e produção dos fatores de virulência. Dessa forma, métodos moleculares também devem ser aplicados (Eaton & Gasson, 2001).

Enterococcus constituem um gênero de BAL conhecidamente patogênico. Muitos estudos mostram que *Enterococcus* são predominantes na microbiota lática autóctone (Dolci et al., 2008b; Dal Bello et al., 2010; Ortolani et al., 2010b) e que apesar de serem capazes de produzir bacteriocinas e diversas substâncias desejáveis para produção de derivados do leite, possuem também diversos genes associados a

fatores de virulência (Herreros et al., 2005; Barbosa et al., 2010; Komprda et al., 2010; Hadji-Sfaxi et al., 2011; Moraes et al., 2012). Apesar de nenhum caso de infecção de origem alimentar por *Enterococcus* ter sido descrita, essas cepas possuem grande capacidade de transferência de genes. De qualquer forma a verificação da presença de fatores de virulência e até mesmo da capacidade de transferência de genes deve ser avaliada antes da utilização dessas cepas em alimentos.

3. Queijos "artesanais"

No Brasil, de acordo com a portaria N° 146 (Brasil, 1996), o leite utilizado para fabricação de queijos deve ser submetido ao tratamento térmico ou se utilizado leite cru, o queijo deve ser maturado por no mínimo 60 dias antes do consumo. Porém em 2013, com a publicação da IN 30, foi permitida a comercialização de queijos artesanais tradicionalmente elaborados a partir de leite cru maturados por um período inferior a 60 (sessenta) dias, desde que estudos técnico-científicos comprovem que a redução do período de maturação não compromete a qualidade e a inocuidade do produto (Brasil, 2013).

Outros países, como França e Itália, possuem uma grande variedade de queijos que são produzidos com leite cru, porém possuem controlados sistemas de produção leiteira, com contaminação microbiana inicial do leite muito mais baixa quando comparado ao leite produzido no Brasil. A microbiota do leite cru representa uma importante parte da microbiota de muitos queijos artesanais conferindo a estes produtos características sensoriais muito específicas (Asteri et al., 2010).

Guerrero et al. (2009) definiram alimentos tradicionais como produtos frequentemente consumidos ou associados com tradições específicas em que seu modo de preparo é normalmente transmitido de uma geração para outra, com precisão e de

acordo com o patrimônio gastronômico, com pouco ou nenhum processamento/manipulação, e são reconhecidos por causa de suas propriedades sensoriais e associados a certa área, local, região ou país.

O queijo Minas artesanal se encaixa perfeitamente nesta definição. Esses queijos são produzidos usando leite de vaca cru, um agente coagulante, e adição do pingo, um fermento láctico natural recolhido a partir do soro que drena do próprio queijo. Esse ingrediente transfere para o queijo as características do solo, clima e vegetação da região (Instituto do Patrimônio Histórico e Artístico Nacional, 2008). Os queijos são classificados de acordo com sua origem de produção e as mais importantes são: Serro, Canastra, Araxá, Cerrado e Campo das Vertentes. Os fatores ambientais de cada uma destas regiões proporciona o desenvolvimento de uma microbiota específica que interfere com as características sensoriais e fornece sabores específicos em cada queijo. Esses queijos são semi-maturados ou maturados de cor branco-creme homogênea, consistência macia, textura lisa e dependendo do tempo de maturação possuem a casca mais ou menos amarelada e a consistência macia tende a mais dura. A maturação dos queijos é de 5-10 dias com variações regionais.

A produção de queijo Minas artesanal no estado de Minas Gerais começou a ser regulamentada a partir de 2002, com uma legislação específica (Minas Gerais, 2002) e, posteriormente, o seu modo de produção foi reconhecida como patrimônio imaterial do IPHAN (Instituto do Patrimônio Histórico e Artístico Nacional, 2008). A forma de produção dos queijos é muito simples, de baixo custo e o produto final possui grande aceitação por parte dos consumidores, possuindo uma grande importância social, econômica e cultural. Apesar disso, a principal preocupação em usar o leite cru para a produção de queijos é a transmissão de micro-organismos patogênicos (Brito et al., 2008; Pinto et al., 2009) e a ocorrência de micro-organismos deterioradores.

Porém, a observação de que queijos produzidos com leite cru possuem melhores características sensoriais do que queijos preparados com leite pasteurizado tem aumentado o interesse em preservar as características dos queijos produzidos com leite cru e estimulado o estudo de novas cepas de BAL que possuam algum interesse tecnológico e que sejam capazes de produzir bacteriocinas (Herreros et al., 2003; Bizani et al., 2008; Franciosi et al., 2009; Nieto-Arribas et al., 2010; Dal Bello et al., 2012).

Ainda, o grande aumento da demanda de consumidores por produtos naturais, sem adição de conservantes químicos ou uso de tratamentos térmicos, fez com que a indústria passasse a explorar o uso de BAL como starters funcionais para produção de queijos. Starters funcionais são culturas que possuam pelo menos uma propriedade funcional inerente, seja tecnológica, nutricional ou relacionada à inocuidade alimentar.

O desenvolvimento e uso de novas culturas starters requer o conhecimento do ecossistema do alimento e a caracterização fisiológica e tecnológica dos micro-organismos predominantes (Dal Bello et al., 2012). A qualidade de produtos lácteos fermentados é bastante influenciada por suas características sensoriais. Por isso, na produção de queijos é importante avaliar se a cepa bacteriana candidata possui algumas propriedades desejáveis, como a capacidade de produção rápida de ácidos, tolerância a diferentes concentrações de NaCl, atividade proteolítica e autolítica e produção de exopolissacarídeos e diacetil (Leroy & De Vuyst, 2004).

4. Biodiversidade e ecologia microbiana

Os principais gêneros de BAL que compõem a microbiota autóctone de leite são *Enterococcus*, *Lactococcus*, *Leuconostoc* e *Streptococcus.* Populações psicrotróficas que se desenvolvem durante o armazenamento a frio incluem *Pseudomonas* e

Acinetobacter spp. (Quigley et al., 2011). A composição da microbiota do leite impacta diretamente o desenvolvimento de derivados lácteos.

Muitos trabalhos tem objetivado o estudo da diversidade e da dinâmica das populações de micro-organismos durante a fabricação e maturação de queijos, o que permite associações entre a ocorrência de determinadas espécies ou cepas bacterianas com características sensoriais específicas nos produtos finais (Zamfir et al., 2006; Dolci et al., 2008b; Dolci et al., 2009; Dal Bello et al., 2010; Morandi et al., 2011).

Tradicionalmente, o conhecimento da diversidade microbiana em queijos é realizado a partir do isolamento de cepas bacterianas do alimento, utilizando meios de cultura seletivos com a sua subsequente identificação fenotípica. Apesar de ser um método relativamente sensível, esse método não permite na maioria das vezes a discriminação entre espécies e entre cepas, e também não permite a identificação das relações filogenéticas entre os isolados obtidos. Dessa forma, o uso de técnicas de PCR baseadas na geração de fingerprintings é muito útil para a determinação das estruturas das comunidades microbianas em diferentes ambientes e para o monitoramento de alterações destas comunidades (Randazzo et al., 2009). Didaticamente, todas essas técnicas podem ser divididas em: i) métodos cultura-dependentes, baseadas no cultivo bacteriano seguido do isolamento e identificação fenotípica e molecular; e ii) métodos cultura-independentes, com extração de DNA ou RNA diretamente do alimento, dispensando o cultivo em meios de cultura (Figura 2).

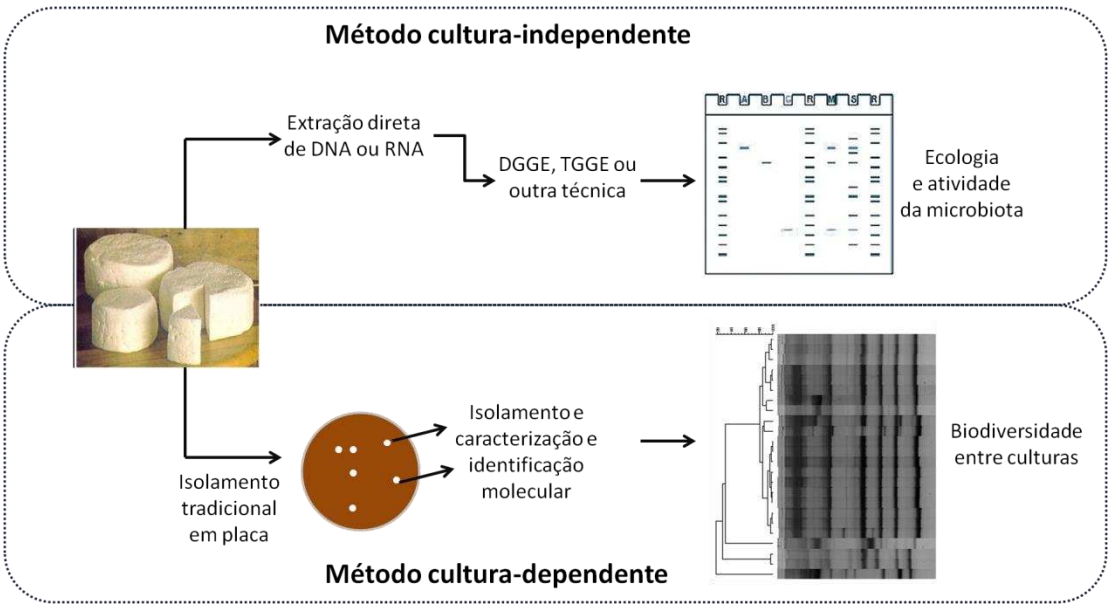

Figura 2. Representação esquemática das abordagens moleculares utilizadas no estudo da ecologia de queijos. Adaptado de Cocolin et al. (2011).

4.1. Métodos cultura-dependentes

Após o cultivo em meios de cultura seletivos e isolamento das colônias de BAL a partir de alimentos, o grande desafio é a identificação dos diversos gêneros e espécies. Na década de 1990, as técnicas moleculares para identificação e caracterização de micro-organismos isolados de alimentos começaram a ser usadas juntamente ou substituindo os testes fenotípicos, morfológicos e bioquímicos. Apesar das duas técnicas muitas vezes chegarem a resultados similares, as técnicas moleculares mostram maior nível de reprodutibilidade, automatismo, rapidez e sensibilidade (Axelsson, 2004; Cocolin et al., 2007; Mohania et al., 2008).

A identificação molecular de BAL baseada no sequenciamento do gene 16S rRNA e na região intergênica entre os genes 16S rRNA e 23S rRNA tem sido cada vez mais aceita e utilizada (Klijn et al., 1991; Ortolani et al., 2010a; Moraes et al., 2013). Os genes 16S e 23S estão presentes em todas as bactérias, são regiões altamente conservadas e possuem variações suficientes para diferenciação entre espécies (Janda &

Abbott, 2007). Vários métodos de identificação são baseados na utilização desses genes, como sondas ribossomais de RNA, PCR espécie-específico, polimorfismo no comprimento de fragmentos de restrição (RFLP), PCR multiplex e TGGE e DGGE associados com o sequenciamento de DNA (Domig et al., 2003; Cocolin et al., 2011).

As cepas isoladas podem também ser caracterizadas e agrupadas de acordo com sua similaridade genética utilizando alguns métodos moleculares, como polimorfismo de DNA amplificado ao acaso (RAPD), amplificação de elementos repetitivos de DNA (rep-PCR) e eletroforese em gel de campo pulsado (PFGE). A análise dos géis gerados por estes métodos se baseia na digitalização dos fingerprintings e consequente produção do dendograma de similaridade. A partir dos dendogramas é possível a identificação de diferentes clusters que fornecem importantes informações sobre a dinâmica e a diversidade entre organismos da mesma espécie (Cocolin et al., 2011).

O método RAPD foi descrito por Williams et al. (1990) e é uma técnica de PCR que se difere do PCR convencional no que diz respeito às condições de amplificação do DNA, ao comprimento do oligonucleotídeo utilizado e a resolução dos produtos obtidos. Os oligonucleotídeos geralmente possuem entre 7-10 pares de bases e não são específicos a qualquer sequência conhecida do genoma bacteriano, determinando anelamentos em sequências ao acaso (Domig et al., 2003). RAPD exibe os polimorfismos existentes na sequência do genoma, permitindo a comparação e diferenciação de cepas bacterianas. O polimorfismo detectado por marcadores RAPD é gerado por mutações ou por rearranjos (inserção ou deleção) no genoma. Diferenças em apenas um par de bases (mutações pontuais) podem ser suficientes para inibir a amplificação. Por essa técnica utilizar condições de amplificação não especificas, como baixa temperatura de anelamento, e consequente baixa reprodutibilidade, outros

métodos são preferidos para serem utilizados para o agrupamento de BAL (Mohammed et al., 2009).

Rep-PCR é um método que se baseia na evidência de que as bactérias contêm cópias exatas de sequências de DNA em seu genoma (Versalovic et al., 1991). Estes elementos repetitivos de DNA, como o REP (palíndromo repetitivo extragênico) são localizados em diferentes regiões do genoma e interrompidos por diferentes distâncias, dependendo da cepa (Versalovic et al., 1992; Domig et al., 2003). A reação de PCR se baseia no uso de oligonucleotídeos que se anelam nas sequências REP e os produtos de DNA gerados serão de diferentes tamanhos, obtidos a partir da amplificação da sequência de DNA localizada entre os elementos repetidos. Os produtos da amplificação são separados de acordo com seu tamanho em eletroforese em gel de agarose. O rep-PCR gera fingerprintings que aparentemente são específicos para cada indivíduo (Versalovic et al., 1991; 1992).

PFGE é uma técnica usada para separação de moléculas de DNA grandes a partir da aplicação de campo elétrico pulsado que periodicamente muda de direção. Os padrões de bandas são gerados a partir da digestão do DNA, utilizando enzimas de restrição. A correta seleção das enzimas de restrição é crucial para realização da técnica, pois os perfis gerados irão depender da sua especificidade à sequência do genoma bacteriano (Randazzo et al., 2009). PFGE tem sido muito usado para diferenciação de bactérias pertencentes a diferentes gêneros, devido ao seu alto poder discriminatório e reprodutibilidade, mas possui algumas limitações, como demorados protocolos para extração e corridas de DNA (Garde et al., 2012).

A utilização de métodos cultura-dependentes para determinação das populações microbianas em sistemas alimentares pode apresentar falhas relacionadas com a capacidade de multiplicação dos micro-organismos em condições laboratoriais específicas. Células estressadas e injuriadas não são identificadas, assim como células

presentes em populações pequenas são frequentemente inibidas por populações microbianas mais numerosas (Hugenholtz et al., 1998; Cocolin et al., 2007). Por estas razões, é fundamental contar com ferramentas que permitem o monitoramento de populações microbianas sem cultivo, o que pode ser conseguido por métodos cultura-independentes.

4.2. Métodos cultura-independentes

Métodos cultura-independentes estão se tornando cada vez mais populares e tem sido muito aplicados para determinação da ecologia microbiana de leite e queijos (Giraffa & Neviani, 2001; Cocolin et al., 2002; Dolci et al., 2008a; Dolci et al., 2009; Giannino et al., 2009; Dolci et al., 2010; Morandi et al., 2011). Essas técnicas permitem a identificação e monitoramento da microbiota do alimento em nível de espécies através da extração de DNA diretamente do alimento, sem necessidade de cultivo em meios de cultura.

Alguns métodos mais utilizados são eletroforese em gel de gradiente desnaturante (DGGE), eletroforese em gel de gradiente de temperatura (TGGE) e hibridização *in situ* por fluorescência (FISH). Essas técnicas possuem as vantagens de serem realizadas mais rapidamente, são mais específicas e sensíveis, permitem a identificação de micro-organismos não cultiváveis, e de células estressadas.

No início da década de 1990, Muyzer et al. (1993) desenvolveram o DGGE com objetivo de caracterizar a microbiota de forma mais rápida e econômica e foi aplicado para o estudo da ecologia microbiana de ecossistemas ambientais, como comunidades de bactérias redutoras de sulfato. Após alguns anos, esta técnica começou a ser utilizada em alimentos fermentados e atualmente existe um grande número de publicações utilizando DGGE em queijos, leite e produtos cárneos (Cocolin et al., 2002;

Cocolin et al., 2004; Cocolin et al., 2007; Dolci et al., 2008a; Dolci et al., 2009; Hu et al., 2009; Dal Bello et al., 2010; Dolci et al., 2010; Kesmen et al., 2011; Morandi et al., 2011).

As técnicas TGGE e DGGE permitem o estudo da diversidade microbiana através da separação eletroforética de pequenos fragmentos de DNA de fita dupla (200 - 700 pb) geradas por uma reação de PCR inicial. Um grampo GC composto de 30 a 40 bases nucleicas é anexado ao final da região 5' do oligonucleotídeo foward utilizado para amplificação do fragmento de DNA de interesse.

No DGGE, o gel de poliacrilamida para a análise dos fragmentos de DNA amplificados possui como agentes desnaturantes as substâncias ureia e formamida, e no TGGE, o gradiente de temperatura é o principal agente desnaturante. Durante a eletroforese, quando a molécula de DNA encontra uma concentração desnaturante de químicos ou temperatura de desnaturação adequada (melting temperature), ocorre desnaturação parcial na dupla fita de DNA (o grampo GC impede a desnaturação completa da dupla-fita de DNA). Cada domínio de desnaturação é sequência-específico, e fragmentos de DNA com tamanhos similares, mas com sequências diferentes são separados de acordo com suas propriedades desnaturantes. A mudança de conformação na estrutura do DNA, causada pela sua desnaturação parcial, reduz a taxa de migração da molécula no gel (Cocolin et al., 2011).

Cada banda que pode ser visualizada nos géis de D/TGGE representa um componente da microbiota. Dessa forma, quanto mais bandas presentes, mais complexa é a microbiota. As bandas ainda podem ser extraídas do gel e após re-amplificação (gene 16S rRNA) podem ser sequenciadas para identificação da espécie microbiana correspondente. Utilizando estes métodos é possível não só obter o perfil das populações microbianas, mas também obter informações sobre a sua dinâmica durante o tempo.

A técnica FISH é muito utilizada para análise de populações microbianas complexas, por exemplo, no solo e sedimentos de lodo. Sua aplicação tem sido muito útil para detecção rápida e específica de patógenos e deteriorantes de alimentos (Cocolin et al., 2007). Ercolini et al. (2003) foram os primeiros a aplicar esta técnica para estudar as comunidades microbianas em alimentos intactos, permitindo a identificação da localização específica das bactérias na matriz do queijo. FISH é uma ferramenta molecular que não é PCR-baseada que emprega sondas de oligonucleotídeos que possuem como alvo RNA ribossomal para rápida e segura detecção e enumeração de micro-organismos específicos através da microscopia de fluorescência (Bottari et al., 2006; Ndoye et al., 2011). Alguns anos depois, Cocolin et al. (2007) otimizaram os protocolos de FISH em amostras de queijos e mostraram que os limites de detecção da técnica era de 10^3 UFC/mL. Com a técnica de FISH, o conhecimento da composição e distribuição dos micro-organismos na matriz do queijo pode ser útil por gerar informações relacionadas a segurança alimentar e determinação de aspectos tecnológicos para a indústria láctea (Ndoye et al., 2011).

Os métodos cultura-independentes surgiram com objetivo de compensar as limitações dos métodos tradicionais cultura-dependentes em relação à determinação da estrutura, diversidade e atividade das comunidades microbianas. Porém, devido a algumas limitações existentes nos métodos moleculares, o ideal seria a combinação no uso dos métodos cultura-dependentes e cultura-independentes.

Referências

Abee, T., Krockel, L., & Hill, C., 1995. Bacteriocins: modes of action and potentials in food preservation and control of food poisoning. *International Journal of Food Microbiology* 28:169.

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

Alkhatib, Z., Abts, A., Mavaro, A., Schmitt, L., & Smits, S.H., 2012. Lantibiotics: how do producers become self-protected? *Journal of Biotechnology* 159:145-154.

Ammor, M.S., Flórez, A.B., & Mayo, B., 2007. Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. *Food Microbiology* 24:559-570.

ANVISA, 1996. Aprova a extensão de uso da nisina com a função de conservador para queijos pasteurizados. *Portaria n. 39.* DOFC 23/01/1996.

Asteri, I.-A., Kittaki, N., & Tsakalidou, E., 2010. The effect of wild lactic acid bacteria on the production of goat's milk soft cheese. *International Journal of Dairy Technology* 63:234-242.

Axelsson, L., 2004. Lactic Acid Bacteria: classification and physiology, p. 01-66. In: Salmnen, S., Von Wright, A., Ouwehand, A. (Ed.), Lactic Acid Bacteria: Microbiological and Functional Aspects. Marcel Dekker Inc, New York.

Barbosa, J., Gibbs, P.A., & Teixeira, P., 2010. Virulence factors among enterococci isolated from traditional fermented meat products produced in the North of Portugal. *Food Control* 21:651-656.

Bizani, D., Morrissy, J.A.C., Dominguez, A.P.M., & Brandelli, A., 2008. Inhibition of *Listeria monocytogenes* in dairy products using the bacteriocin-like peptide cerein 8A. *International Journal of Food Microbiology* 121:229-233.

Bonetta, S., Bonetta, S., Carraro, E., Rantsiou, K., & Cocolin, L., 2008. Microbiological characterisation of Robiola di Roccaverano cheese using PCR–DGGE. *Food Microbiology* 25:786-792.

Borrero, J., Jiménez, J.J., Gútiez, L., Herranz, C., Cintas, L.M., & Hernández, P.E., 2011. Protein expression vector and secretion signal peptide optimization to drive the production, secretion, and functional expression of the bacteriocin enterocin A in lactic acid bacteria. *Journal of Biotechnology* 156:76-86.

Bottari, B., Ercolini, D., Gatti, M., & Neviani, E., 2006. Application of FISH technology for microbiological analysis: current state and prospects. *Applied Microbiology and Biotechnology* 73:485-494.

Bover-Cid, S. & Holzapfel, W.H., 1999. Improved screening procedure for biogenic amine production by lactic acid bacteria. *International Journal of Food Microbiology* 53:33-41.

Brasil, 1996. Regulamentos técnicos de identidade e qualidade dos produtos lácteos. D.O.U. 11/03/1996.

Brasil, 2000. Regulamento técnico de produção, identidade e qualidade do leite de cabra. D.O.U. 08/11/2000.

Brasil. 2013. Instrução Normativa 30. Queijos artesanais manufaturados a partir de leite cru. D.O.U. August 8th 2013.

Brito, J.R.F., Santos, E.M., Arcuri, E.F., Lange, C.C., Brito, M.A., Souza, G.N., Cerqueira, M.M., Beltran, J.M.S., Call, J.E., & Liu, Y., 2008. Retail survey of Brazilian milk and minas frescal cheese and a contaminated dairy plant to establish prevalence, relatedness, and sources of *Listeria monocytogenes* isolates. *Applied and Environmental Microbiology* 74:4954-4961.

Bromberg, R., Moreno, I., Delboni, R.R., Cintra, H.C., & Oliveira, P.T.V., 2005. Characteristics of the bacteriocin produced by *Lactococcus lactis* subsp. *cremoris* CTC 204 and the effect of this compound on the mesophilic bacteria associated with raw beef. *World Journal of Microbiology and Biotechnology* 21:351-358.

Castellano, P., Belfiore, C., Fadda, S., & Vignolo, G., 2008. A review of bacteriocinogenic lactic acid bacteria used as bioprotective cultures in fresh meat produced in Argentina. *Meat Science* 79:483-499.

Chen, H. & Hoover, D., 2006. Bacteriocins and their food applications. *Comprehensive Reviews in Food Science and Food Safety* 2:82-100.

Clementi, F. & Aquilanti, L., 2011. Recent investigations and updated criteria for the assessment of antibiotic resistance in food lactic acid bacteria. *Anaerobe* 17:394-398.

Cocolin, L., Aggio, D., Manzano, M., Cantoni, C., & Comi, G., 2002. An application of PCR-DGGE analysis to profile the yeast populations in raw milk. *International Dairy Journal* 12:407-411.

Cocolin, L., Rantsiou, K., Iacumin, L., Urso, R., Cantoni, C., & Comi, G., 2004. Study of the ecology of fresh sausages and characterization of populations of lactic acid bacteria by molecular methods. *Applied and Environmental Microbiology* 70:1883-1894.

Cocolin, L., Diez, A., Urso, R., Rantsiou, K., Comi, G., Bergmaier, I., & Beimfohr, C., 2007. Optimization of conditions for profiling bacterial populations in food by culture-independent methods. *International Journal of Food Microbiology* 120:100-109.

Cocolin, L., Dolci, P., & Rantsiou, K., 2011. Biodiversity and dynamics of meat fermentations: the contribution of molecular methods for a better comprehension of a complex ecosystem. *Meat Science* 89:296-302.

Coton, M., Romano, A., Spano, G., Ziegler, K., Vetrana, C., Desmarais, C., Lonvaud-Funel, A., Lucas, P., & Coton, E., 2010. Occurrence of biogenic amine-forming lactic acid bacteria in wine and cider. *Food Microbiology* 27:1078-1085.

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

Courvalin, P., 2006. Vancomycin resistance in gram-positive cocci. *Clinical Infectious Diseases* 42:S25-S34.

D'Angelis, C.E.M., Polizello, A.C.M., Nonato, M.C., Spadaro, A.C.C., & de Martinis, E.C.P., 2009. Purification, characterization and n-terminal amino acid sequencing of sakacin 1, a bacteriocin produced by *Lactobacillus sakei* 1. *Journal of Food Safety* 29:636-649.

Dal Bello, B., Rantsiou, K., Bellio, A., Zeppa, G., Ambrosoli, R., Civera, T., & Cocolin, L., 2010. Microbial ecology of artisanal products from North West of Italy and antimicrobial activity of the autochthonous populations. *LWT - Food Science and Technology* 43:1151-1159.

Dal Bello, B., Cocolin, L., Zeppa, G., Field, D., Cotter, P.D., & Hill, C., 2012. Technological characterization of bacteriocin producing *Lactococcus lactis* strains employed to control *Listeria monocytogenes* in Cottage cheese. *International Journal of Food Microbiology* 153:58-65.

De Kwaadsteniet, M., Ten Doeschate, K., & Dicks, L., 2008. Characterization of the structural gene encoding nisin F, a new lantibiotic produced by a *Lactococcus lactis* subsp. *lactis* isolate from freshwater catfish (*Clarias gariepinus*). *Applied and Environmental Microbiology* 74:547-549.

de Martinis, E.C.P., Alves, V.F., & Franco, B.D.G.M., 2002. Fundamentals and perspectives for the use of bacteriocins produced by lactic acid bacteria in meat products. *Food Reviews International* 18:191-208.

de Vos, W.M., Kuipers, O.P., van der Meer, J.R., & Siezen, R.J., 1995. Maturation pathway of nisin and other lantibiotics: post-translationally modified antimicrobial peptides exported by Gram-positive bacteria. *Molecular Microbiology* 17:427-437.

De Vuyst, L. & Leroy, F., 2007. Bacteriocins from lactic acid bacteria: production, purification, and food applications. *Journal of Molecular Microbiology and Biotechnology* 13:194-199.

Deegan, L.H., Cotter, P.D., Hill, C., & Ross, P., 2006. Bacteriocins: Biological tools for bio-preservation and shelf-life extension. *International Dairy Journal* 16:1058-1071.

Dolci, P., Alessandria, V., Rantsiou, K., Rolle, L., Zeppa, G., & Cocolin, L., 2008a. Microbial dynamics of Castelmagno PDO, a traditional Italian cheese, with a focus on lactic acid bacteria ecology. *International Journal of Food Microbiology* 122:302-311.

Dolci, P., Alessandria, V., Zeppa, G., Rantsiou, K., & Cocolin, L., 2008b. Microbiological characterization of artisanal Raschera PDO cheese: Analysis of its indigenous lactic acid bacteria. *Food Microbiology* 25:392-399.

Dolci, P., Barmaz, A., Zenato, S., Pramotton, R., Alessandria, V., Cocolin, L., Rantsiou, K., & Ambrosoli, R., 2009. Maturing dynamics of surface microflora in Fontina PDO cheese studied by culture-dependent and -independent methods. *Journal of Applied Microbiology* 106:278–287.

Dolci, P., Alessandria, V., Rantsiou, K., Bertolino, M., & Cocolin, L., 2010. Microbial diversity, dynamics and activity throughout manufacturing and ripening of Castelmagno PDO cheese. *International Journal of Food Microbiology* 143:71-75.

Domig, K.J., Mayer, H.K., & Kneifel, W., 2003. Methods used for the isolation, enumeration, characterisation and identification of *Enterococcus* spp.: 2. Pheno-and genotypic criteria. *International Journal of Food Microbiology* 88:165-188.

Eaton, T.J. & Gasson, M.J., 2001. Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. *Applied and Environmental Microbiology* 67:1628-1635.

Ercolini, D., Hill, P.J., & Dodd, C.E.R., 2003. Development of a fluorescence in situ hybridization method for cheese using a 16S rRNA probe. *Journal of Microbiological Methods* 52:267-271.

FAOSTAT, F.A., 2014.http://faostat.fao.org/site/569/DesktopDefault.aspx?PageID=569 (accessed 01/10/2014).

Field, D., Connor, P.M., Cotter, P.D., Hill, C., & Ross, R.P., 2008. The generation of nisin variants with enhanced activity against specific gram-positive pathogens. *Molecular Microbiology* 69:218-230.

Franciosi, E., Settanni, L., Cavazza, A., & Poznanski, E., 2009. Biodiversity and technological potential of wild lactic acid bacteria from raw cows' milk. *International Dairy Journal* 19:3-11.

Franz, C.M.A.P. & Holzapfel, W.H., 2004. The genus *Enterococcus*: biotechnological and safety issues, p. 199– 248. In: Salminen, S., von Wright, A., and Ouwehand, A. (Eds.), Lactic Acid Bacteria: Microbiological and Functional Aspects. Marcel Dekker, Inc, New York.

Fukao, M., Obita, T., Yoneyama, F., Kohda, D., Zendo, T., Nakayama, J., & Sonomoto, K., 2008. Complete covalent structure of nisin Q, new natural nisin variant, containing post-translationally modified amino acids. *Bioscience, Biotechnology, and Biochemistry* 72:1750-1755.

Garde, S., Gaya, P., Arias, R., & Nuñez, M., 2012. Enhanced PFGE protocol to study the genomic diversity of *Clostridium* spp. isolated from Manchego cheeses with late blowing defect. *Food Control* 28:392-399.

Giannino, M.L., Marzotto, M., Dellaglio, F., & Feligini, M., 2009. Study of microbial diversity in raw milk and fresh curd used for Fontina cheese production by culture-independent methods. *International Journal of Food Microbiology* 130:188-195.

Giraffa, G., Pepe, G., Locci, F., Neviani, E., & Carminati, D., 1995. Hemolytic activity, production of thermonuclease and biogenic amines by dairy enterococci. *Italian Journal of Food Science* 7:341-349.

Giraffa, G. & Neviani, E., 2001. DNA-based, culture-independent strategies for evaluating microbial communities in food-associated ecosystems. *International Journal of Food Microbiology* 67:19-34.

Guerrero, L., Guàrdia, M.D., Xicola, J., Verbeke, W., Vanhonacker, F., Zakowska-Biemans, S., Sajdakowska, M., Sulmont-Rosse, C., Issanchou, S., & Contel, M., 2009. Consumer-driven definition of traditional food products and innovation in traditional foods. A qualitative cross-cultural study. *Appetite* 52:345-354.

Hadji-Sfaxi, I., El-Ghaish, S., Ahmadova, A., Batdorj, B., Le Blay-Laliberté, G., Barbier, G., Haertlé, T., & Chobert, J.-M., 2011. Antimicrobial activity and safety of use of *Enterococcus faecium* PC4. 1 isolated from Mongol yogurt. *Food Control* 22:2020-2027.

Haenlein, G.F.W., 2004. Goat milk in human nutrition. *Small Ruminant Research* 51:155-163.

Hendrickx, A., Willems, R.J.L., Bonten, M.J.M., & van Schaik, W., 2009. LPxTG surface proteins of enterococci. *Trends in Microbiology* 17:423-430.

Herreros, M.A., Fresno, J.M., González Prieto, M.J., & Tornadijo, M.E., 2003. Technological characterization of lactic acid bacteria isolated from Armada cheese (a Spanish goats' milk cheese). *International Dairy Journal* 13:469-479.

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

Hu, P., Zhou, G., Xu, X., Li, C., & Han, Y., 2009. Characterization of the predominant spoilage bacteria in sliced vacuum-packed cooked ham based on 16S rDNA-DGGE. *Food Control* 20:99-104.

Hugenholtz, P., Goebel, B.M., & Pace, N.R., 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *Journal of Bacteriology* 180:4765-4774.

Instituto do Patrimônio Histórico e Artístico Nacional, 2008. Patrimônio imaterial. Bens registrados. Modo artesanal de fazer Queijo de Minas, nas regiões do Serro e das serras da Canastra e do Salitre.

Janda, J.M. & Abbott, S.L., 2007. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *Journal of Clinical Microbiology* 45:2761-2764.

Jandal, J.M., 1996. Comparative aspects of goat and sheep milk. *Small Ruminant Research* 22:177-185.

Jenness, R., 1980. Composition and characteristics of goat milk: review. *Journal of Dairy Science* 63:1605-1630.

Kesmen, Z., Yetiman, A.E., Gulluce, A., Kacmaz, N., Sagdic, O., Cetin, B., Adiguzel, A., Sahin, F., & Yetim, H., 2011. Combination of culture-dependent and culture-independent molecular methods for the determination of lactic microbiota in sucuk. *Internationa Journal of Food Microbiology* 15:428-435.

Klaenhammer, T.R., 1988. Bacteriocins of lactic acid bacteria. *Biochimie* 70:337-349.

Klijn, N., Weerkamp, A.H., & de Vos, W.M., 1991. Identification of mesophilic lactic acid bacteria by using polymerase chain reaction-amplified variable regions of 16S rRNA and specific DNA probes. *Applied and Environmental Microbiology* 57:3390-3393.

Komprda, T., Sladkova, P., Petirova, E., Dohnal, V., & Burdychova, R., 2010. Tyrosine-and histidine-decarboxylase positive lactic acid bacteria and enterococci in dry fermented sausages. *Meat Science* 86:870-877.

Landete, J.M., de las Rivas, B., Marcobal, A., & Muñoz, R., 2007. Molecular methods for the detection of biogenic amine-producing bacteria on foods. *International Journal of Food Microbiology* 117:258-269.

Leroy, F. & De Vuyst, L., 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends in Food Science & Technology* 15:67-78.

Levy, S.B. & Marshall, B., 2004. Antibacterial resistance worldwide: causes, challenges and responses. *Nature Medicine* 10:S122-S129.

Lopes, M.F.S., Simões, A.P., Tenreiro, R., Marques, J.J.F., & Crespo, M.T.B., 2006. Activity and expression of a virulence factor, gelatinase, in dairy enterococci. *International Journal of Food Microbiology* 112:208-214.

Mathur, S. & Singh, R., 2005. Antibiotic resistance in food lactic acid bacteria - A review. *International Journal of Food Microbiology* 105:281-295.

McAuliffe, O., Ross, R.P., & Hill, C., 2001. Lantibiotics: structure, biosynthesis and mode of action. *FEMS Microbiology Reviews* 25:285-308.

Minas Gerais, 2002. Regulamento da lei nº 14.185, de 31 de janeiro de 2002 que dispõe sobre o processo de produção de queijo minas artesanal. Minas Gerais Executive Daily.

Mohammed, M., Abd El-Aziz, H., Omran, N., Anwar, S., Awad, S., & El-Soda, M., 2009. Rep-PCR characterization and biochemical selection of lactic acid bacteria isolated from the Delta area of Egypt. *International Journal of Food Microbiology* 128:417-423.

Mohania, D., Nagpal, R., Kumar, M., Bhardwaj, A., Yadav, M., Jain, S., Marotta, F., Singh, V., Parkash, O., & Yadav, H., 2008. Molecular approaches for identification and characterization of lactic acid bacteria. *Journal of Digestive Diseases* 9:190-198.

Montel, M.-C., Buchin, S., Mallet, A., Delbes-Paus, C., Vuitton, D.A., Desmasures, N., & Berthier, F., 2014. Traditional cheeses: Rich and diverse microbiota with associated benefits. *International Journal of Food Microbiology* 177:136-154.

Moraes, P.M., Perin, L.M., Todorov, S.D., Silva, A., Jr., Franco, B.D.G.M., & Nero, L.A., 2012. Bacteriocinogenic and virulence potential of *Enterococcus* isolates obtained from raw milk and cheese. *Journal of Applied Microbiology* 113:318-328.

Moraes, P.M., Perin, L.M., Silva Jr., A., & Nero, L.A., 2013. Comparison of phenotypic and molecular tests to identify lactic acid bacteria. *Brazilian Journal of Microbiology* 44:109-112.

Morandi, S., Brasca, M., & Lodi, R., 2011. Technological, phenotypic and genotypic characterisation of wild lactic acid bacteria involved in the production of Bitto PDO Italian cheese. *Dairy Science & Technology* 91:341-359.

Mulders, J.W., Boerrigter, I.J., Rollema, H.S., Siezen, R.J., & Vos, W.M., 1991. Identification and characterization of the lantibiotic nisin Z, a natural nisin variant. *European Journal of Biochemistry* 201:581-584.

Muyzer, G., De Waal, E.C., & Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* 59:695-700.

Ndoye, B., Rasolofo, E.A., LaPointe, G., & Roy, D., 2011. A review of the molecular approaches to investigate the diversity and activity of cheese microbiota. *Dairy Science & Technology* 91:495-524.

Nero, L.A., Mattos, M.R., Barros, M.A.F., Ortolani, M.B.T., Beloti, V., & Franco, B.D.G.M., 2008. *Listeria monocytogenes* and *Salmonella* spp. in raw milk produced in Brazil: occurrence and interference of indigenous microbiota in their isolation and development. *Zoonoses and Public Health* 55:299-305.

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

Ortolani, M.B.T., Moraes, P.M., Perin, L.M., Viçosa, G.N., Carvalho, K.G., Silva Jr., A., Sesma, F., Franco, B.D.G.M., & Nero, L.A., 2010a. Molecular identification of naturally occurring bacteriocinogenic and bacteriocinogenic-like lactic acid bacteria in raw milk and soft cheese. *Journal of Dairy Science* 93:2880-2886.

Ortolani, M.B.T., Yamazi, A.K., Moraes, P.M., Viçosa, G.N., & Nero, L.A., 2010b. Microbiological quality and safety of raw milk and soft cheese and detection of autochthonous lactic acid bacteria with antagonistic activity against *Listeria monocytogenes*, *Salmonella* spp., and *Staphylococcus aureus*. *Foodborne Pathogens and Disease* 7:175-180.

Park, Y.W., 2007. Rheological characteristics of goat and sheep milk. *Small Ruminant Research* 68:73-87.

Perin, L.M., Moraes, P.M., Viçosa, G.N., Silva Júnior, A., & Nero, L.A., 2012. Identification of bacteriocinogenic *Lactococcus* isolates from raw milk and cheese capable of producing nisin A and nisin Z. *International Dairy Journal* 25:46-51.

Pingitore, E.V., Todorov, S.D., Sesma, F., & Franco, B.D.G.M., 2012. Application of bacteriocinogenic *Enterococcus mundti* CRL35 and *Enterococcus faecium* ST88Ch in the control of *Listeria monocytogenes* in fresh minas cheese. *Food Microbiology* 32:38-47.

Pinto, M.S., de Carvalho, A.F., Pires, A.C.d.S., de Paula, J.C.J., Sobral, D., & Magalhães, F.A.R., 2009. Survival of *Listeria innocua* in Minas traditional Serro cheese during ripening. *Food Control* 20:1167-1170.

Piper, C., Hill, C., Cotter, P.D., & Ross, R.P., 2011. Bioengineering of a Nisin A producing *Lactococcus lactis* to create isogenic strains producing the natural variants Nisin F, Q and Z. *Microbial Biotechnology* 4:375-382.

Quigley, L., O'Sullivan, O., Beresford, T.P., Ross, R.P., Fitzgerald, G.F., & Cotter, P.D., 2011. Molecular approaches to analysing the microbial composition of raw milk and raw milk cheese. *International Journal of Food Microbiology* 150:81-94.

Randazzo, C.L., Caggia, C., & Neviani, E., 2009. Application of molecular approaches to study lactic acid bacteria in artisanal cheeses. *Journal of Microbiological Methods* 78:1-9.

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

Rodríguez, E., González, B., Gaya, P., Nuñez, M., & Medina, M., 2000. Diversity of bacteriocins produced by lactic acid bacteria isolated from raw milk. *International Dairy Journal* 10:7-15.

Rogers, L. & Whittier, E., 1928. Limiting factors in the lactic fermentation. *Journal of Bacteriology* 16:211.

Rollema, H.S., Metzger, J.W., Both, P., Kuipers, O.P., & Siezen, R.J., 1996. Structure and biological activity of chemically modified nisin A species. *European Journal of Biochemistry* 241:716-722.

Saini, A.L. & Gill, R.S., 1991. Goat milk: An attractive alternate. *Indian Dairyman* 42:562-564.

Settanni, L., Franciosi, E., Cavazza, A., Cocconcelli, P.S., & Poznanski, E., 2011. Extension of Tosèla cheese shelf-life using non-starter lactic acid bacteria. *Food Microbiology* 28:883-890.

Todorov, S.D., Prévost, H., Lebois, M., Dousset, X., LeBlanc, J.G., & Franco, B.D.G.M., 2011. Bacteriocinogenic *Lactobacillus plantarum* ST16Pa isolated from papaya (*Carica papaya*) - from isolation to application: characterization of a bacteriocin. *Food Research International* 44:1351-1363.

Toomey, N., Bolton, D., & Fanning, S., 2010. Characterisation and transferability of antibiotic resistance genes from lactic acid bacteria isolated from Irish pork and beef abattoirs. *Research in Microbiology* 161:127-135.

Versalovic, J., Koeuth, T., & Lupski, R., 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerpriting of bacterial genomes. *Nucleic Acids Research* 19:6823-6831.

Versalovic, J., Koeuth, T., Zhang, Y.-H., McCabe, E.R., & Lupski, J.R., 1992. Quality control for bacterial inhibition assays: DNA fingerprinting of microorganisms by rep-PCR. *Screening* 1:175-183.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., & Tingey, S.V., 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* 18:6531-6535.

Wirawan, R.E., Klesse, N.A., Jack, R.W., & Tagg, J.R., 2006. Molecular and genetic characterization of a novel nisin variant produced by *Streptococcus uberis*. *Applied and Environmental Microbiology* 72:1148-1156.

Zamfir, M., Vancanneyt, M., Makras, L., Vaningelgem, F., Lefebvre, K., Pot, B., Swings, J., & De Vuyst, L., 2006. Biodiversity of lactic acid bacteria in Romanian dairy products. *Systematic and Applied Microbiology* 29:487-495.

Zhang, Z.-G., Ye, Z.-Q., Yu, L., & Shi, P., 2011. Phylogenomic reconstruction of lactic acid bacteria: an update. *BMC Evolutionary Biology* 11:1.

OBJETIVOS

Objetivo Geral

Caracterizar o potencial bacteriocinogênico e fatores de virulência de BAL naturalmente presentes em leite de cabra cru, e avaliar o seu potencial bioconservador para produção de queijo Minas utilizando leite de cabra cru.

Objetivos Específicos

- Caracterizar a diversidade molecular dos grupos de BAL que compõem a microbiota autóctone do leite de cabra cru;
- Caracterizar o potencial bacteriocinogênico das cepas de BAL isoladas de leite cru de cabra;
- Realizar a identificação molecular de BAL antagonistas através do sequenciamento dos genes 16S rRNA e *phe*S;
- Realizar a pesquisa de fatores de virulência, resistência a antibióticos e produção de aminas biogênicas (AB) por BAL bacteriocinogênicas isoladas de leite cru de cabra, utilizando métodos fenotípicos e moleculares;
- Produção de queijo Minas utilizando leite de cabra cru e uma cepa de BAL (*Lactococcus lactis* subsp. *lactis* GLc05) produtora de nisina e ausência de fatores de virulência;
- Avaliar a interferência de *L. lactis* subsp. *lactis* GLc05 na dinâmica microbiana após a produção dos queijos Minas e durante a maturação;
- Quantificar a concentração de AB presentes nos queijos Minas.

CAPÍTULO 1. Antagonistic lactic acid bacteria isolated from goat milk and identification of a novel nisin variant *Lactococcus lactis*

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Title page

Antagonistic lactic acid bacteria isolated from goat milk and identification of a novel nisin variant *Lactococcus lactis*

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Abstract

Background

The raw goat milk microbiota is considered a good source of novel bacteriocinogenic lactic acid bacteria (LAB) strains that can be exploited as an alternative for use as biopreservatives in foods. The constant demand for such alternative tools justifies studies that investigate the antimicrobial potential of such strains.

Results

The obtained data identified a predominance of *Lactococcus* and *Enterococcus* strains in raw goat milk microbiota with antimicrobial activity against *Listeria monocytogenes* ATCC 7644. Enzymatic assays confirmed the bacteriocinogenic nature of the antimicrobial substances produced by the isolated strains, and PCR reactions detected a variety of bacteriocin-related genes in their genomes. Rep-PCR identified broad genetic variability among the *Enterococcus* isolates, and close relations between the *Lactococcus* strains. The sequencing of PCR products from *nis*-positive *Lactococcus* allowed the identification of a predicted nisin variant not previously described and possessing a wide inhibitory spectrum.

Conclusions

Raw goat milk was confirmed as a good source of novel bacteriocinogenic LAB strains, having identified *Lactococcus* isolates possessing variations in their genomes that suggest the production of a nisin variant not yet described and with potential for use as biopreservatives in food due to its broad spectrum of action

Background

Goat milk is the second variety of milk most produced in the world [1]. Their production is increasing mainly because it could be an alternative to substitute the consumption of cow milk, due to evidences that it does not induce allergies, presents high digestibility, and also possess high nutritional quality [2]. As cow milk, goat milk has a very rich and complex autochthonous microbiota, and its detailed knowledge is essential for a future use of this matrix for the production of fermented products [3,4]. The main responsible for the natural fermentation of these products are microorganisms from the Lactic Acid Bacteria (LAB) group, that are widely studied due to their potential use as adjuvants and biopreservatives in foods [3,5–8]. Many studies already demonstrated that BAL has considerable inhibitory activity against pathogenic and spoilage microorganisms in foods [7–12], mainly by the production of bacteriocins [13,14].

Bacteriocins are small proteins that present antimicrobial activity and are of particular interest to food industries, representing natural alternatives to improve the safety and quality of foods [13,15]. Considering these characteristics, new bacteriocinogenic LAB strains and their bacteriocins are continuously searched, however only nisin and pediocin PA-1 are the bacteriocins allowed to be applied in food, including cheeses [15,16]. Nisin is a lantibiotic produced by some *Lactococcus lactis* strains and up to now five nisin variants are already known: nisin A (the first to be discovered), Z, Q, U and F [17–19]. The differences between these variants are based on the changes in the amino acid chain, what could interfere in their antimicrobial activity.

The main sources of novel LAB strains capable of producing bacteriocins are food systems, mainly ones that are naturally contaminated with a diversity of

microorganisms, such as animal origin products [9,20,21]. The production characteristics of meat and dairy products facilitate contamination by distinct microbial groups, determining a rich autochthonous microbiota in such food products. In this context, the autochthonous microbiota of raw goat milk is particularly interesting due to its diversity and the presence of several bacteriocinogenic LAB strains, as observed in previous studies [4,5,22,23]. Once isolated from a food sample, the antimicrobial activity of bacterocinogenic LAB strains must be properly characterized [24]. Relevant information that must be investigated includes possible bacteriocins the strains are able to produce, which can be assessed by the identification of specific genes related to known bacteriocins, followed by sequencing to identify variants [25,26]. Additionally, it is important to verify the inhibitory spectrum of the bacteriocins produced by newly isolated LAB strains. Such data can justify further studies with purified bacteriocins, in order to check a diversity of characteristics that allow their use in the food industry as biopreservatives.

The present study aimed to characterize the diversity of the main LAB groups that compose the autochthonous microbiota of raw goat milk and their bacteriocinogenic potential, in order to identify novel strains capable of producing known bacteriocin variants with potential application as biopreservatives.

Methods

Samples and microbiological analysis

Raw goat milk samples were collected from 11 goat farms (two samples per farm) located in Viçosa, Minas Gerais state, Brazil, and subjected to ten-fold dilution using 0.85 % NaCl (w/v). Selected dilutions were pour plated in duplicate and in distinct culture media: M17 (Oxoid Ltd., Basingstoke, England, incubated at 35°C for 48 h, and

at 42°C for 48 h), de Man, Rogosa and Sharpe (MRS) (Oxoid, incubated at 30°C for 48 h, under anaerobic conditions using GasPak EZ^{TM} Gas Generating Container Systems, BD - Becton, Dickinson and Co., Franklin Lakes, NJ, USA), MRS at pH 5.5 (Oxoid, incubated at 35°C for 48 h, under anaerobic conditions using GasPak, BD), and Kanamycin Aesculin Azide (Oxoid, incubated at 35°C for 48 h). After incubation, colonies were enumerated and the results expressed as log colony-forming units per mL (log cfu/mL). From each culture media and sample, representative colonies were selected (about 10 % of the observed count) and subjected to Gram staining and checked for catalase production. LAB characteristic colonies were subjected to addition microbiological analysis as described in the following sections.

Antimicrobial activity and bacteriocin production

Isolates identified as LAB (Gram positive and catalase negative) were subjected to the spot-on-the-lawn method to identify their antimicrobial activity against *Listeria monocytogenes* ATCC 7644, according to CB Lewus, A Kaiser and TJ Montville [27]. Briefly, LAB isolates were cultured in MRS broth (Oxoid) at 35°C for 24 h, after which 1 μL aliquots were spotted on the surface of MRS agar (Oxoid) and incubated at 25°C for 24 h under anaerobic conditions (GasPak, BD); then, brain heart infusion (BHI, Oxoid) broth was added to bacteriological agar at 0.8 % (w/v) and *L. monocytogenes* ATCC 7644 at 10^5 cfu/mL was overlaid and incubated at 35°C for 24 h. The presence of inhibition halos was recorded as the antimicrobial activity of the tested isolate.

Isolates that presented antimicrobial activity were subjected to the spot-on-the-lawn protocol [27,28] to identify the bacteriocinogenic nature of their antimicrobial substances. For this, after the first incubation of the tested isolates in MRS plates, 2 mm diameter wells were cut adjacent to the colonies and enzyme solutions at 20 mg/mL

were added: α-chimotrypsin, proteinase K, TPCK trypsin, α-amylase, papain, *Streptomyces griseus* protease, *Aspergillus niger* lipase, and lysozyme (all from Sigma-Aldrich, Inc., St. Louis, MO, USA). Half-moon halos associated with proteases were indicative of the bacteriocinogenic nature of the antimicrobial substances produced by the tested isolates.

Molecular identification and rep-PCR fingerprinting of bacteriocinogenic isolates

Bacteriocinogenic isolates were cultured in MRS broth (Oxoid) at 35°C for 12 h, and the obtained cultures were subjected to DNA extraction using the Genomic Wizard DNA Purification Kit (Promega Corp., Madison, WI, USA). Identification of these isolates was done by sequencing their 16S rRNA genes using the primers P1V1 and P4V3 (Table 1). Isolates identified as *Enterococcus* spp. were identified at the species level by sequencing the *pheS* (phenylalanyl-tRNA synthase α-subunit) gene using the primers pheS-21 and pheS-22 (Table 1). PCR reactions consisted of 25 μL of Go Taq Green Master Mix 2x (Promega), 10 pMol of each pair of primers, 2 μL of DNA (50 ng/μL) and ultra pure PCR water (Promega) to a final volume of 50 μL. PCR conditions for 16S rRNA were as described by N Klijn, AH Weerkamp and WM de Vos [29], and for *pheS* as detailed by SM Naser, FL Thompson, B Hoste, D Gevers, P Dawyndt, M Vancanneyt and J Swings [30], using the annealing temperatures described in Table 1. The PCR products (Table 1) were double-strand sequenced by Macrogen Inc. (Seoul, Korea), and the identification was given only for sequences with 100 % of similarity when compared to the database of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/genbank) using the software Basic Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov/blast.cgi).

Table 1 Primers sequences, annealing temperatures, and expected fragment sizes of PCR reactions targeting specific genes for identification and bacteriocins encoding genes

Target	primers sequences (5'-sequence-3')	fragment size (bp)	annealing	reference
16S rRNA	GCGGCGTGCCTAATACATGC	700	42°C	[29]
	ATCTACGCATTTCACCGCTAC			
*phe*S	CAYCCNGCHCGYGAYATGC	470	46°C	[30]
	CCWARVCCRAARGCAAARCC			
*lan*B	TATGATCGAGAARYAKAWAGATATGG	400-500	40°C	[17,19]
	TTATTAIRCAIATGIAYDAWACT			
*lan*C	TAATTTAGGATWISYIMAYGG	200-300	40°C	[17,19]
	ACCWGKIIIICCRTRRCACCA			
*lan*M	ATGCWAGWYWTGCWCATGG	200-300	40°C	[17,19]
	CCTAATGAACCRTRRYAYCA			
*nis*A	GGATAGTATCCATGTCTG	250	55°C	[31]
	CAATGATTTCGTTCGAAG			
*ent*A	CATCATCCATAACTATATTTG	126	56°C	[32]
	AAATATTATGGAAATGGAGTGTAT			
*ent*B	GAAAATGATCACAGAATGCCTA	162	58°C	[32]
	GTTGCATTTAGAGTATACATTTG			
*ent*P	TATGGTAATGGTGTTTATTGTAAT	120	58°C	[32]
	ATGTCCCATACCTGCCAAAC			
*ent*L50AB	STGGGAGCAATCGCAAAATTAG	98	56°C	[32]
	ATTGCCCATCCTTCTCCAAT			
*ent*AS48	GAGGAGTITCATGATTTAAAGA	340	56°C	[32]
	CATATTGTTAAATTACCAAGCAA			

Rep-PCR was performed according the protocol described by B Dal Bello, K Rantsiou, A Bellio, G Zeppa, R Ambrosoli, T Civera and L Cocolin [9] using a single primer $(GTG)_5$ (5'-GTGGTGGTGGTGGTG-3'). PCR reactions contained 12.5 μL of Go Taq Green Master Mix 2x (Promega), 50 pMol of the primer, 2 μL of DNA (50 ng/μL) and ultra pure PCR water (Promega) to a final volume of 25 μL. PCR conditions were: 1) 5

min at 95°C, (2) 30 cycles of 30 s at 95°C; 30 s at 40°C and 8 min at 65°C, and (3) final extension of 16 min at 65°C. PCR products were electrophoresed in 2 % (w/v) agarose gels for 6 h at a constant voltage of 75 V, in 0.5 × Tris/Borate/EDTA buffer (TBE). Gels were stained using GelRed (Biotium Inc., Hayward, CA, USA), and recorded using a transilluminator LPIX (Loccus Biotecnologia, São Paulo, SP, Brazil). Fingerprints were analysed using BioNumerics 4.6 (Applied Maths, Kortrijk, Belgium): The similarities among profiles were calculated using the Pearson correlation. Dendograms were constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

Bacteriocin encoding genes

Bacteriocinogenic isolates were subjected to PCR to detect genes related to the expression of lantibiotics (*lan*B, *lan*C, and *lan*M), nisin (*nis*), and enterocins (A, P, B, L50A, L50B, and AS-48) using the primers presented in Table 1. PCR reactions consisted of 12.5 μL of Go Taq Green Master Mix 2x (Promega), 100 pMol of lantibiotics primers, or 60 pMol of nisin primers, or 10 pMol of enterocins primers, 1 μL of DNA (200 ng/μL), and ultra pure PCR water (Promega) to a final volume of 25 μL. All PCR reactions were conducted according the following conditions: 1) 95 °C for 5 min, 2) 30 cycles at 95 °C for 1 min, annealing temperature (Table 1) for 1 min, and 72 °C for 1 min, and 3) final extension at 72 °C for 10 min. The PCR products were electrophoresed in 1 % (w/v) agarose gels in 0.5 × TBE, and stained in a GelRed bath (Biotium). Fragments with the specific expected sizes (Table 1) were recorded as positive results for each bacteriocin-encoding gene for each isolate. Positive results were confirmed by repeating the PCR reactions.

Nisin gene sequencing and inhibitory spectrum of nisin positive isolates

PCR products of *nis*-positive isolates were sequenced by Macrogen Inc. The obtained results were analysed using the software Sequencher™ 4.1.4 (Technology Drive, Ann Arbor, MI, USA) in order to identify similarities between the translated amino-acid sequences and a nisin A, Z, Q, F or U sequences previously deposited in GenBank. In addition, nisin-positive isolates were subjected to the spot-on-the-lawn protocol, as described previously [27], to identify their inhibitory activity against 22 target strains: 4 LAB, 4 *Listeria* spp., 2 *Pseudomonas* spp., 4 *Salmonella* spp., 6 *Staphylococcus* spp. and 2 *E. coli*. The diameters of the inhibition halos were measured to characterize the antimicrobial activities of the tested isolates.

Results and discussion

LAB counts of raw goat milk

The obtained mean counts for LAB of goat milk samples obtained by distinct culture media considered in the present study are presented in Table 2. The mean counts ranged from 3.07 to 3.89 log cfu/mL, and a total of 682 colonies was selected from the plated culture media, among which 423 were characterized as possessing typical LAB characteristics (Table 2). The majority of isolates from the LAB collection was characterized as cocci (377), a group described as the predominant component of raw milk microbiota [21,33]. The obtained results also highlighted the absence of adequate selectivity in the employed culture media, even for LAB (Table 2), necessitating further phenotypic analysis for proper characterization of the isolates [34]. The autochthonous microbiota of the goat milk could have originated mainly from utensils and environmental conditions, being highly influenced by the hygienic procedures of milking [35–37]. The method of storage also has a direct impact on the microbiota of raw milk, high temperatures being determinant for the predominance of lactococci [33].

Table 2 Mean counts and numbers of obtained isolates from distinct culture media used to enumerate presumptive lactic acid bacteria (LAB) groups from raw goat milk samples, and their typical LAB characteristics, antimicrobial activity, and sensitivity to eight distinct enzymatic solutions

results	**Group**	**culture media (incubation condition)**[a]					**total**
		M17 (35°C)	MRS (pH 5.5)	KAA	M17 (42°C)	MRS	
mean count (log cfu/mL)		3.89	3.47	3.07	3.65	3.61	-
obtained isolates (n)	--	134	138	142	128	140	682
typical LAB	Gram positive cocci, catalase negative	57	79	108	46	87	377
	Gram positive bacilli, catalase negative	7	18	4	5	12	46
antimicrobial activity[b]	--	13	4	23	7	10	57
enzymatic sensitivity[b]	α-chimotrypsin	9	2	13	7	6	37
	proteinase K	11	1	18	6	10	46
	TPCK trypsin	10	3	10	5	10	38
	α-amylase	3	0	1	0	3	7
	papain	4	3	8	3	6	24
	Streptomyces griseus protease	13	4	18	4	10	49
	Aspergillus niger lipase	9	2	6	4	7	28
	lysozyme	1	0	1	0	0	2

[a] MRS: de Man, Rogosa and Sharpe; KAA: Kanamycin Aesculin Azide.

[b] identified by spot-on-the-lawn method [27] using *Listeria monocytogenes* ATCC 7644 as target.

Antimicrobial activity and bacteriocin production

From the LAB collection obtained from raw goat milk, 57 isolates presented antimicrobial activity against *L. monocytogenes* ATCC 7644 (Table 2). This foodborne pathogen was selected as a target because previous studies have demonstrated its susceptibility to the antimicrobial substances produced by LAB; it is usually adopted as an indicator of such activity [11,22,25,38,39].

The bacteriocinogenic activity was confirmed by the enzymatic assays in 54 of the 57 antagonistic isolates (Table 2). These isolates produced antimicrobial substances that were degraded by distinct enzymes solutions, mainly by proteinase K and *Streptomyces griseus* protease. The sensitivity to proteases indicated the proteinaceous nature of the produced substances, typical for bacteriocins [13,40]. In addition, the observed results indicated that the bactericions produced by the tested isolates would be degraded by pancreatic enzymes and they would not interfere with the intestinal microbiota of the consumer [41].

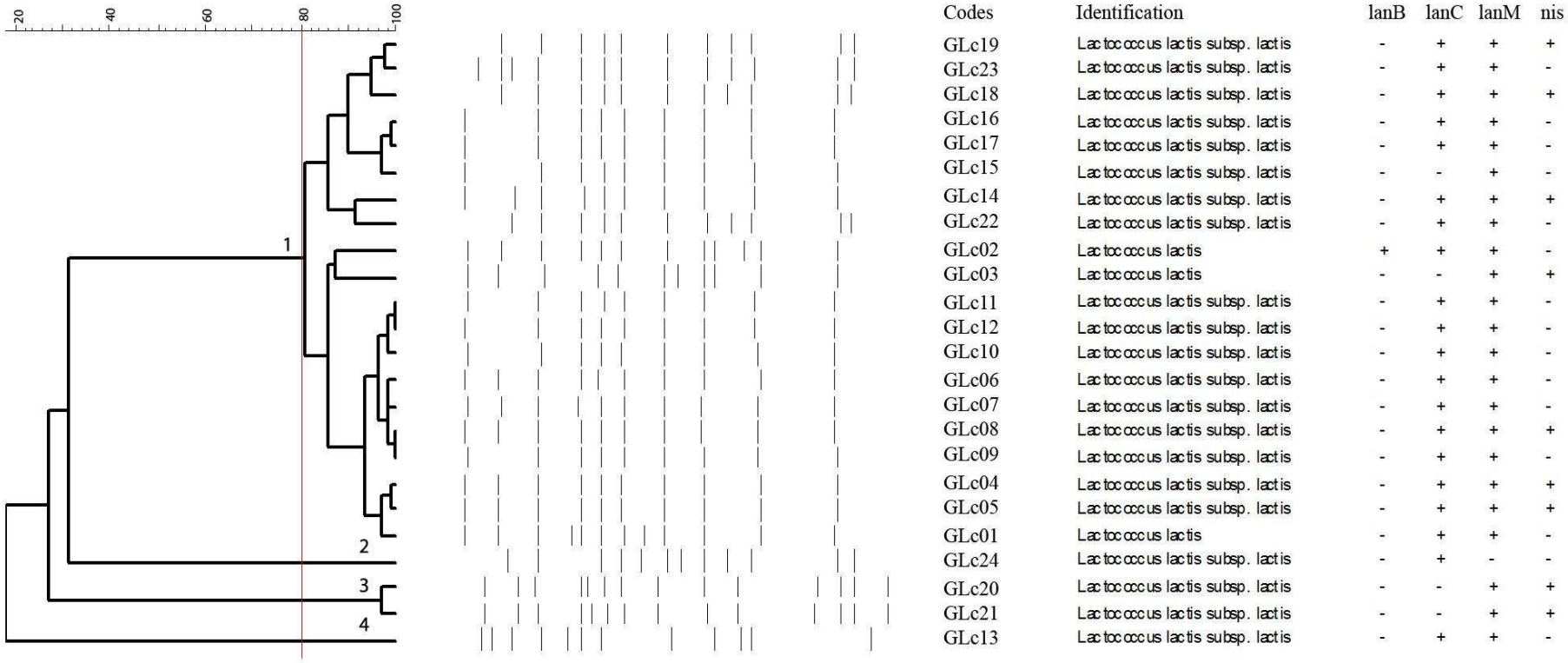
It was also observed that some isolates produced antimicrobial substances with sensitivities to α-amylase (7) and lypase (28), suggesting the presence of carbohydrates and lipids in their structures [42,43]. These substances can interfere with bacteriocins stability, demanding further studies to verify their appropriateness as biopreservatives in foods [44].

Molecular identification and rep-PCR fingerprinting of bacteriocinogenic isolates

All 57 isolates that presented antimicrobial activity against *L. monocytogenes* ATCC 7644, whether they produced antimicrobial substances sensitive to enzymes or not

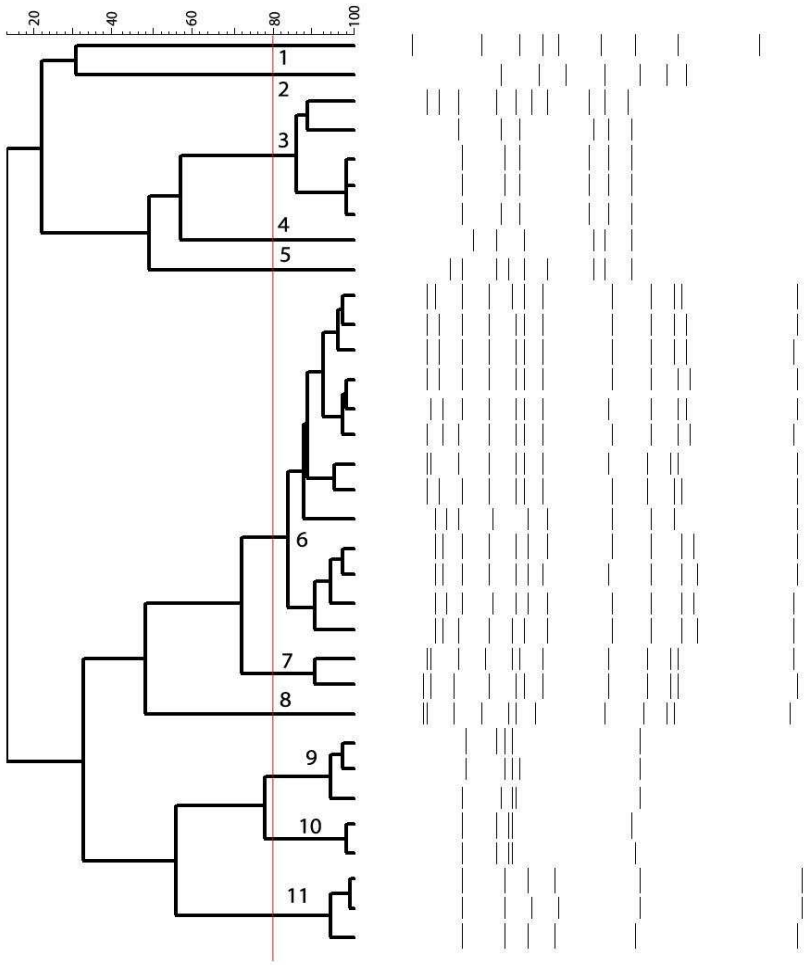
(Table 2), was subjected to molecular identification and rep-PCR fingerprinting. The isolates were identified as *Lactococcus* spp. (24 isolates: 21 *L. lactis* subsp. *lactis*, and 3 *L. lactis*) and *Enterococcus* spp. (33 isolates: 17 *E. durans*, 8 *E. faecalis*, 7 *E. faecium*, and 1 *E. hirae*). For *Lactococcus* spp., it was observed that sequencing of the V1 region (90 bp) of the 16S rRNA gene was sufficient to provide a proper and reliable identification of the isolates, with variations that allowed differentiation of their species and subspecies [29]. However, sequencing of the same region in *Enterococcus* spp. isolates was not enough to provide a reliable identification at the species level, as observed in previous studies [45–48]; this limitation demanded sequencing of the *phe*S gene for a proper identification [30]. Considering the obtained results, isolates from raw goat milk that presented antimicrobial activity were identified as *Lactococcus* spp. and *Enterococcus* spp., as is usually observed in studies that investigate this activity in autochthonous microbiota from food systems [9,11,49].

For rep-PCR fingerprinting analysis, the isolates were grouped considering their genus identification and 80 % similarity to the obtained profiles (Figures 1 and 2). *Lactococcus* spp. isolates were grouped in four clusters, being 20 strains comprising in only one cluster, demonstrating large homology between them (Figure 1). For *Enterococcus*, the isolates were grouped in 11 clusters, demonstrating their biodiversity and evident similarities between isolates from the same species (Figure 2). Rep-PCR has already been described as a reliable methodology to determine the intra-species biodiversity of LAB isolated from foods, and also to assess the genetic variability of bacteriocinogenic strains [9,50,51].



Codes	Identification	lanB	lanC	lanM	nis
GLc19	Lactococcus lactis subsp. lactis	-	+	+	+
GLc23	Lactococcus lactis subsp. lactis	-	+	+	-
GLc18	Lactococcus lactis subsp. lactis	-	+	+	+
GLc16	Lactococcus lactis subsp. lactis	-	+	+	-
GLc17	Lactococcus lactis subsp. lactis	-	+	+	-
GLc15	Lactococcus lactis subsp. lactis	-	-	+	-
GLc14	Lactococcus lactis subsp. lactis	-	+	+	+
GLc22	Lactococcus lactis subsp. lactis	-	+	+	-
GLc02	Lactococcus lactis	+	+	+	-
GLc03	Lactococcus lactis	-	-	+	+
GLc11	Lactococcus lactis subsp. lactis	-	+	+	-
GLc12	Lactococcus lactis subsp. lactis	-	+	+	-
GLc10	Lactococcus lactis subsp. lactis	-	+	+	-
GLc06	Lactococcus lactis subsp. lactis	-	+	+	-
GLc07	Lactococcus lactis subsp. lactis	-	+	+	-
GLc08	Lactococcus lactis subsp. lactis	-	+	+	+
GLc09	Lactococcus lactis subsp. lactis	-	+	+	-
GLc04	Lactococcus lactis subsp. lactis	-	+	+	+
GLc05	Lactococcus lactis subsp. lactis	-	+	+	+
GLc01	Lactococcus lactis	-	+	+	-
GLc24	Lactococcus lactis subsp. lactis	-	+	-	-
GLc20	Lactococcus lactis subsp. lactis	-	-	+	+
GLc21	Lactococcus lactis subsp. lactis	-	-	+	+
GLc13	Lactococcus lactis subsp. lactis	-	+	+	-

Figure 1 Dendrogram generated after cluster analysis of rep-PCR fingerprints of bacteriocinogenic *Lactococcus* spp. obtained from raw goat milk. Clusters are indicated by numbers. Presence (+) or absence (-) of bacteriocin encoding genes are also indicated.



Codes	Identification	lanB	lanC	lanM	nis	entA	entP	entB	entAS48	entL50AB
GEn02	Enterococcus durans	+	-	+	-	-	-	-	-	+
GEn33	Enterococcus hirae	-	+	+	+	-	-	-	-	+
GEn26	Enterococcus faecium	-	-	+	-	-	+	-	-	+
GEn32	Enterococcus faecium	-	-	-	-	-	+	-	-	+
GEn30	Enterococcus faecium	-	-	+	+	-	+	-	-	+
GEn31	Enterococcus faecium	-	-	+	-	-	+	-	-	+
GEn29	Enterococcus faecium	-	-	+	-	-	+	-	-	+
GEn28	Enterococcus faecium	-	+	-	-	-	+	-	-	+
GEn27	Enterococcus faecium	-	+	+	+	-	+	+	-	+
GEn07	Enterococcus durans	+	-	+	+	-	-	-	-	+
GEn08	Enterococcus durans	+	-	-	-	-	-	-	-	+
GEn09	Enterococcus durans	+	-	+	-	-	-	-	-	+
GEn10	Enterococcus durans	+	-	+	-	-	-	-	-	+
GEn11	Enterococcus durans	+	-	+	-	-	-	-	-	-
GEn12	Enterococcus durans	+	-	-	-	-	-	-	-	+
GEn04	Enterococcus durans	+	-	+	-	-	-	-	-	+
GEn06	Enterococcus durans	+	-	+	-	-	-	-	-	+
GEn17	Enterococcus durans	+	-	+	-	-	-	-	-	+
GEn14	Enterococcus durans	+	-	-	-	-	-	-	-	+
GEn16	Enterococcus durans	+	-	+	-	-	-	-	-	+
GEn13	Enterococcus durans	+	-	+	-	-	+	-	-	+
GEn15	Enterococcus durans	+	-	-	+	-	-	-	-	+
GEn03	Enterococcus durans	+	-	-	-	-	-	-	-	-
GEn05	Enterococcus durans	-	-	-	-	-	-	-	-	+
GEn01	Enterococcus durans	+	-	+	-	-	-	-	-	+
GEn24	Enterococcus faecalis	-	+	-	+	-	+	-	-	+
GEn25	Enterococcus faecalis	-	+	-	+	-	-	-	-	-
GEn21	Enterococcus faecalis	-	+	-	+	-	-	-	-	+
GEn18	Enterococcus faecalis	-	+	-	-	-	-	-	-	-
GEn19	Enterococcus faecalis	-	+	-	+	-	+	-	-	+
GEn22	Enterococcus faecalis	-	+	-	+	-	-	-	-	+
GEn23	Enterococcus faecalis	-	-	-	-	-	-	-	-	+
GEn20	Enterococcus faecalis	-	+	-	-	-	-	-	-	+

Figure 2 Dendrogram generated after cluster analysis of rep-PCR fingerprints of bacteriocinogenic *Enterococcus* spp. obtained from raw goat milk. Clusters are indicated by numbers. Presence (+) or absence (-) of bacteriocin encoding genes are also indicated.

Bacteriocin encoding genes

Figures 1 and 2 also present the results for bacteriocin encoding genes assessed in the *Lactococcus* spp. and *Enterococcus* spp. isolates, respectively.

All *Lactococcus* spp. isolates presented lantibiotic genes in distinct associations, only one (GLc02) presenting *lan*B, *lan*C and *lan*M simultaneously (Figure 1). *lan*B was the less frequent gene, while *lan*C and *lan*M usually were present simultaneously in the majority of isolates; this result was expected, since both genes are located in the same operon in the bacterial genome [52]. However, the isolated presence of *lan*C or *lan*M has already been described in previous studies [19,25]. For *Enterococcus* isolates, 30 isolates presented at least one of the tested lantibiotic genes; no isolates presented *lan*B, *lan*C and *lan*M simultaneously (Figure 2). Cytolisin is a class I lantibiotic produced by *Enterococcus* spp., a bacteriocin that can be related to the tested genes [53]. Considering the antimicrobial potential of the isolates, the presence of at least one of the tested genes would be sufficient for lantibiotic production [17,19].

A lower frequency of positive results was observed for *nis* in the tested *Lactococcus* isolates (9 strains) compared to similar studies identifying the bacteriocinogenic potential of this genus (Figure 1) [9,22,25,49]. Still considering the results for the *nis* gene, ten *Enterococcus* isolates presented typical PCR amplification products (Figure 2). The occurrence of *Enterococcus* strains possessing nisin-related genes has already been reported, and can be explained by the capability of this genus to acquire new genetic elements [40]. However, positive results for the *nis* gene must not be related to the production of nisin by *Enterococcus* isolates.

No *Enterococcus* isolates presenting encoded genes for enterocin A and enterocin AS-48 (Figure 2). Only a single isolate (GEn27) presented a positive result for the enterocin

B gene, and 10 isolates, from five distinct clusters, for the enterocin P gene (Figure 2). Enterocin A and enterocin P are bacteriocins classified in subclass IIa (pediocin-like bacteriocins), with typical high inhibitory activity against *Listeria* spp. (Javed et al. 2011). The enterocin L50AB gene was detected in 29 isolates, from all identified genetic profiles (Figure 2); this bacterocin is classified in subclass IIb, characterized by its synthesis without leader peptides and demanding a complex system for transport [54,55].

The three LAB isolates that presented antimicrobial activity but an absence of enzymatic sensitivity in their produced substances (Table 2) were two *Lactococcus* (GLc20 and GLc21) and one *Enterococcus* (GEn27) (Figures 1 and 2). However, the three isolates presented positive results for bacteriocin-related genes, indicating that they were unable to express them. When assessing the bacteriocingeonic potential and activity of LAB, the absence of production of bacteriocins by gene positive strains is a common finding, since bacteriocin production is mediated by a diversity of genetic and environmental factors [13,40].

Nisin gene sequencing and inhibitory spectrum of nisin positive isolates

The nine *Lactocccus* isolates that presented positive results for *nis* were identified as capable of producing a novel nisin variant. Their amino-acid sequence were diverse from to the other nisin variants already described (Figure 3). In all translated sequences the typical variation in nisin Z was identified: an asparagine instead of a histidine in position 27 (Figure 3), as described previously [25,56]. In addition, all isolates presented identical variations in their translated sequences when compared to a reference sequences of nisin (Figure 3): 1) in the leader peptide, an aspartic acid was replaced by an asparagine in position -7 ; 2) except for GLc03, an isoleucine was

replaced by a valine in position +4; and 3) a leucine was replaced by a valine in position +16 (Figure 3). Concerning the nisin leader peptide sequence, in the position -7, one negative-charged amino-acid (aspartic acid) was replaced by one uncharged amino-acid (asparagine). This same replacement also occurs in Nisin U1 (Figure 3). Indicating that this change cannot interfere with the correct activity of the peptide. It is important to highlight two characteristics: 1) variations in the sequence between positions -18 and -15 would interfere with nisin production, and 2) mutagenesis in Arg^{1-} and Ala^{4-} would affect cleavage of the leader peptide, resulting in a non-active nisin [52]. However, the observed modification in the leader peptide of the translated sequences was not in these regions, indicating that nisin production and activity would not be affected in the tested isolates (Figure 3). Considering the mature peptide, in positions +4 and +16 of the nisin sequence, one neutral amino-acid (isoleucine and leucine respectively) was replaced by other neutral amino-acid (valine). The only described modification in the +4 region is in nisin U (isoleucine replaced by lysine) [19]. The last variation and well know is in position +27, where one uncharged amino-acid (asparagine) is replaced by one positive electrically charge and basic amino-acid (histidin). This typical change for nisin Z was previously described as responsible for increasing its inhibitory spectrum due to its better diffusion capacity in culture media. It is common to observe variations in the amino-acid sequences of lantibiotics, including nisin, that then require proper characterization since they can interfere with the antimicrobial activity of these substances [18]. The observed variations in the translated nisin sequences have not been reported before, after consulting GenBank.

Reference	GenBank number	Sequence of structural peptide
		-20 · -10 · +1 · +10 · +20 · +30
Nisin A	L16226.1	M S T K D F N L D L V S V S K K D - S G A S P R I T S I S L C T P G C K T G A L M G C N M K T A T C H C S I H V S K
Nisin Z	X61144.1	M S T K D F N L D L V S V S K K D - S G A S P R I T S I S L C T P G C K T G A L M G C N M K T A T C N C S I H V S K
Nisin Q	AB362350.1	M S T K D F N L D L V S V S K T D - S G A S T R I T S I S L C T P G C K T G V L M G C N L K T A T C N C S V H V S K
Nisin U1	DQ146939.1	M N N E D F N L D L I K I S K E N N S G A S P R I T S K S L C T P G C K T G I L M T C P L K T A T C G C H F G - - -
Nisin U2	GU384321.1	M S T K D F N L D L V S V S K K D - S G A S P R V T S K S L C T P G C K T G I L T G C P L K T A T C G C H F G - - -
Nisin F	EU057979.1	M S T K D F N L D L V S V S K K D - S G A S P R I T S I S L C T P G C K T G A L M G C N M K T A T C N C S V H V S K
Wild Strains		
GLc03	KF146295	M S T K D F N L D L V S V S K K N - S G A S P R I T S I S L C T P G C K T G A V M G C N M K T A T C N C S I H V S K
GLc04	KF146296	M S T K D F N L D L V S V S K K N - S G A S P R I T S V S L C T P G C K T G A V M G C N M K T A T C N C S I H V S K
GLc05	KF146297	M S T K D F N L D L V S V S K K N - S G A S P R I T S V S L C T P G C K T G A V M G C N M K T A T C N C S I H V S K
GLc08	KF146298	M S T K D F N L D L V S V S K K N - S G A S P R I T S V S L C T P G C K T G A V M G C N M K T A T C N C S I H V S K
GLc14	KF146299	M S T K D F N L D L V S V S K K N - S G A S P R I T S V S L C T P G C K T G A V M G C N M K T A T C N C S I H V S K
GLc18	KF146300	M S T K D F N L D L V S V S K K N - S G A S P R I T S V S L C T P G C K T G A V M G C N M K T A T C N C S I H V S K
GLc19	KF146301	M S T K D F N L D L V S V S K K N - S G A S P R I T S V S L C T P G C K T G A V M G C N M K T A T C N C S I H V S K
GLc20	KF146302	M S T K D F N L D L V S V S K K N - S G A S P R I T S V S L C T P G C K T G A V M G C N M K T A T C N C S I H V S K
GLc21	KF146303	M S T K D F N L D L V S V S K K N - S G A S P R I T S V S L C T P G C K T G A V M G C N M K T A T C N C S I H V S K

Figure 3 Amino-acid sequences from the already described nisin variants and from the nine nisin positive *Lactococcus* spp. strains obtained from raw goat milk. The leader peptide is composed by 23 amino-acids, followed by amino-acids representing the mature peptide. Amino-acids highlighted in grey indicate variations when compared to the references. The complete amino-acid sequences have been deposited in GenBank (accession numbers KF146295 to KF146303, respectively).

Table 3 shows the inhibitory activity of the *nis* positive *Lactococcus* isolates against several microbial targets. It can be observed that the isolates presented inhibitory activity mainly against the tested Gram positive bacteria, and lower frequencies of inhibition against Gram negative bacteria. These results indicate that the bacteriocins produced by the tested LAB isolates have interesting antimicrobial activities, highlighting the relevance of raw goat milk as a source of bacteriocinogenic strains [23]. In addition, the obtained results indicate that the variations in nisin structure predicted in the present study (Figure 3) did not affect the antimicrobial activity of the isolates. Considering the main characteristics of bacteriocins, the inhibitory activity against the tested Gram negative bacteria must be due to non-specific antimicrobial substances produced by the LAB strains, such as organic acids or peroxide [24,34].

Table 3 Inhibitory activity (diameters of inhibition halos, mm) of *nis* positive *Lactococcus* isolates obtained from raw goat milk against target microorganisms, identified by the spot-on-the-lawn methodology

target genus	species/serotype	origin*	*nis* positive isolates								
			GLc04	GLc05	GLc08	GLc14	GLc18	GLc19	GLc20	GLc21	GLc03
Lactobacillus	*L. sakei*	ATCC 15521	11	13	9	9	5	11	0	0	5
Lactococcus	*L. lactis* subsp. *lactis*	ATCC 7962	11	9	8	7	0	7	0	0	0
	L. lactis subsp. *lactis*	13Lc23, wild strain, present study	13	11	11	11	0	12	0	0	7
	L. lactis subsp. *lactis*	2En4, wild strain, present study	13	11	11	7	7	10	7	7	7
Listeria	*L. monocytogenes*	ATCC 7644	11	11	11	9	15	13	7	7	9
	L. monocytogenes	ATCC 15313	9	9	7	7	0	7	7	5	10
	L. monocytogenes	60, wild strain, beef origin	15	14	12	9	7	13	5	5	5
	L. innocua	76, wild strain, beef origin	5	5	5	5	5	5	5	5	9
Staphylococcus	*S. aureus*	ATCC 12598	9	7	7	7	7	5	7	7	7
	S. aureus	ATCC 14458	9	7	7	7	7	9	11	7	7
	S. aureus	ATCC 29213	8	7	7	7	7	7	9	0	7
	S. aureus	27AF1, wild strain, cheese origin	9	9	9	7	5	11	7	0	9
	S. aureus	27ST1, wild strain, cheese origin	9	9	9	7	5	7	11	7	9
	S. aureus	26BP6, wild strain, cheese origin	13	13	14	7	7	13	7	0	7
Escherichia	*E. coli*	ATCC 11229	0	0	0	0	0	0	0	0	0
	E. coli	ATCC 00171	0	0	0	0	0	0	0	0	0
Pseudomonas	*P. aeruginosa*	ATCC 27853	5	5	5	5	0	0	5	0	0
	P. fluorescens	ATCC 10038	5	5	5	0	0	0	0	0	0
Salmonella	*S.* Typhimurium	ATCC 14028	7	7	5	5	0	0	0	0	0
	S. Cholerasuis	38, wild strain, beef origin	0	0	0	0	0	0	0	0	0
	S. Enteritidis	258, wild strain, poultry origin	7	7	7	5	5	5	5	5	0
	S. Typhi	40, wild strain, beef origin	0	0	0	0	0	0	0	0	0

* ATCC: American Type Culture Collection, Manassas, VA.

Conclusions

In conclusion, the present study highlighted the diversity of LAB in the raw goat milk microbiota, representing a potential source of novel bacteriocinogenic strains to be further studied concerning their antimicrobial activity. In addition, *Lactococcus* strains were identified as possessing variations in their *nis* gene sequences that would result in production of a nisin variant not yet described, and also possessing a wide inhibitory spectrum.

Availability of supporting data

The amino-acid and nucleotide sequences for nisin gene from positive *Lactococcus* spp. strains were deposited and available in the GenBank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/genbank). The accession numbers are KF146295 - KF146303.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LMP: AB, MT, ES, FG. LAN: AB, MT, ES, FG. Both authors read and approved the final manuscript.

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References

1. Food and Agriculture Organization of the United Nations. http://faostat3.org/faostat-gateway/go/to/download/Q/QI/E.

2. Haenlein G: **Goat milk in human nutrition.** *Small Ruminant Res* 2004, **51**(2)**:**155–163.

3. Asteri I, Kittaki N, Tsakalidou E: **The effect of wild lactic acid bacteria on the production of goat's milk soft cheese.** *Int J Dairy Technol* 2010, **63**(2)**:**234–242.

4. Psoni L, Kotzamanidis C, Yiangou M, Tzanetakis N, Litopoulou-Tzanetaki E: **Genotypic and phenotypic diversity of *Lactococcus lactis* isolates from Batzos, a Greek PDO raw goat milk cheese.** *Int J Food Microbiol* 2007, **114**(2)**:**211–220.

5. Colombo E, Franzetti L, Frusca M, Scarpellini M: **Phenotypic and genotypic characterization of lactic acid bacteria isolated from artisanal italian goat cheese.** *J Food Prot* 2010, **73**(4)**:**657–662.

6. Nikolic M, Terzic-Vidojevic A, Jovcic B, Begovic J, Golic N, Topisirovic L: **Characterization of lactic acid bacteria isolated from Bukuljac, a homemade goat's milk cheese.** *Int J Food Microbiol* 2008, **122**(1)**:**162–170.

7. Perin L, Miranda R, Camargo A, Colombo M, Carvalho A, Nero L: **Antimicrobial activity of the Nisin Z producer *Lactococcus lactis* subsp. *lactis* Lc08 against *Listeria monocytogenes* in skim milk.** *Arq Bras Med Vet Zootec* 2013, **65**(5)**:**1554–1560.

8. Pingitore EV, Todorov SD, Sesma F, Franco BDGM: **Application of bacteriocinogenic *Enterococcus mundtii* CRL35 and *Enterococcus faecium* ST88Ch in the control of *Listeria monocytogenes* in fresh Minas cheese.** *Food Microbiol* 2012, **32**(1)**:**38–47.

9. Dal Bello B, Rantsiou K, Bellio A, Zeppa G, Ambrosoli R, Civera T, Cocolin L: **Microbial ecology of artisanal products from North West of Italy and antimicrobial activity of the autochthonous populations.** *LWT - Food Sci Technol* 2010, **43**(7)**:**1151–1159.

10. Nero LA, Mattos MR, Barros MAF, Ortolani MBT, Beloti V, Franco BDGM: ***Listeria monocytogenes* and *Salmonella* spp. in raw milk produced in Brazil: occurrence and interference of indigenous microbiota in their isolation and development.** *Zoonoses Public Health* 2008, **55**(6)**:**299–305.

11. Ortolani MBT, Moraes PM, Perin LM, Viçosa GN, Carvalho KG, Silva A Jr, Sesma F, Franco BDGM, Nero LA: **Molecular identification of naturally occurring bacteriocinogenic and bacteriocinogenic-like lactic acid bacteria in raw milk and soft cheese.** *J Dairy Sci* 2010, **93**(7)**:**2880–2886.

12. Munoz-Atienza E, Gomez-Sala B, Araujo C, Campanero C, del Campo R, Hernandez P, Herranz C, Cintas L: **Antimicrobial activity, antibiotic**

susceptibility and virulence factors of Lactic Acid Bacteria of aquatic origin intended for use as probiotics in aquaculture. *BMC Microbiol* 2013, **13**(1)**:**15.

13. Cotter PD, Hill C, Ross PR: **Bacteriocins: developing innate immunity for food.** *Nat Rev Microbiol* 2005, **3**(10)**:**777–788.

14. Leroy F, De Vuyst L: **Lactic acid bacteria as functional starter cultures for the food fermentation industry.** *Trends Food Sci Technol* 2004, **15**(2)**:**67–78.

15. Castellano P, Belfiore C, Fadda S, Vignolo G: **A review of bacteriocinogenic lactic acid bacteria used as bioprotective cultures in fresh meat produced in Argentina.** *Meat Sci* 2008, **79**(3)**:**483–499.

16. De Vuyst L, Leroy F: **Bacteriocins from lactic acid bacteria: production, purification, and food applications.** *J Mol Microbiol Biotechnol* 2007, **13**(4)**:**194–199.

17. Hyink O, Balakrishnan M, Tagg JR: ***Streptococcus rattus*** **strain BHT produces both a class I two-component lantibiotic and a class II bacteriocin.** *FEMS Microbiol Lett* 2006, **252**(2)**:**235–241.

18. McAuliffe O, Ross RP, Hill C: **Lantibiotics: structure, biosynthesis and mode of action.** *FEMS Microbiol Rev* 2001, **25**(3)**:**285–308.

19. Wirawan RE, Klesse NA, Jack RW, Tagg JR: **Molecular and genetic characterization of a novel nisin variant produced by *Streptococcus uberis*.** *Appl Environ Microbiol* 2006, **72**(2)**:**1148–1156.

20. Franciosi E, Settanni L, Cavazza A, Poznanski E: **Biodiversity and technological potential of wild lactic acid bacteria from raw cows' milk.** *Int Dairy J* 2009, **19**(1)**:**3–11.

21. Ortolani MBT, Yamazi AK, Moraes PM, Viçosa GN, Nero LA: **Microbiological quality and safety of raw milk and soft cheese and detection of autochthonous lactic acid bacteria with antagonistic activity against *Listeria monocytogenes*, *Salmonella* spp., and *Staphylococcus aureus*.** *Foodborne Pathog Dis* 2010, **7**(2)**:**175–180.

22. Rodríguez E, González B, Gaya P, Nuñez M, Medina M: **Diversity of bacteriocins produced by lactic acid bacteria isolated from raw milk.** *Int Dairy J* 2000, **10**(1)**:**7–15.

23. Schirru S, Todorov SD, Favaro L, Mangia NP, Basaglia M, Casella S, Comunian R, Franco BDGM, Deiana P: **Sardinian goat's milk as source of bacteriocinogenic potential protective cultures.** *Food Control* 2012, **25**(1)**:**309–320.

24. Deegan LH, Cotter PD, Hill C, Ross P: **Bacteriocins: Biological tools for bio-preservation and shelf-life extension.** *Int Dairy J* 2006, **16**(9)**:**1058–1071.

25. Perin LM, Moraes PM, Viçosa GN, Silva Júnior A, Nero LA: **Identification of bacteriocinogenic *Lactococcus* isolates from raw milk and cheese capable of producing nisin A and nisin Z.** *Int Dairy J* 2012, **25**(1)**:**46–51.

26. Kruger MF, Barbosa MS, Miranda A, Landgraf M, Destro MT, Todorov SD, Franco BDGM: **Isolation of bacteriocinogenic strain of *Lactococcus lactis* subsp. *lactis* from Rocket salad (*Eruca sativa* Mill.) and evidences of production of a variant of nisin with modification in the leader-peptide.** *Food Control* 2013, **33**(2)**:**467–476.

27. Lewus CB, Kaiser A, Montville TJ: **Inhibition of food-borne bacterial pathogens by bacteriocins from lactic acid bacteria isolated from meat.** *Appl Environ Microbiol* 1991, **57**(6)**:**1683–1688.

28. Tagg JR, Dajani AS, Wannamaker LW: **Bacteriocins of gram-positive bacteria.** *Bacteriol Rev* 1976, **40**(3)**:**722.

29. Klijn N, Weerkamp AH, de Vos WM: **Identification of mesophilic lactic acid bacteria by using polymerase chain reaction-amplified variable regions of 16S rRNA and specific DNA probes.** *Appl Environ Microbiol* 1991, **57**(11)**:**3390–3393.

30. Naser SM, Thompson FL, Hoste B, Gevers D, Dawyndt P, Vancanneyt M, Swings J: **Application of multilocus sequence analysis (MLSA) for rapid identification of *Enterococcus* species based on *rpo*A and *phe*S genes.** *Microbiology* 2005, **151**(7)**:**2141–2150.

31. Li H, O'Sullivan DJ: **Heterologous expression of the *Lactococcus lactis* bacteriocin, nisin, in a dairy *Enterococcus* strain.** *Appl Environ Microbiol* 2002, **68**(7)**:**3392–3400.

32. Toit MD, Franz CMAP, Dicks LMT, Holzapfel WH: **Preliminary characterization of bacteriocins produced by *Enterococcus faecium* and *Enterococcus faecalis* isolated from pig faeces.** *J Appl Microbiol* 2001, **88**(3)**:**482–494.

33. Wouters J, Ayad EHE, Hugenholtz J, Smit G: **Microbes from raw milk for fermented dairy products.** *Int Dairy J* 2002, **12**(2)**:**91–109.

34. Carr FJ, Chill D, Maida N: **The lactic acid bacteria: a literature survey.** *Crit Rev Microbiol* 2002, **28**(4)**:**281–370.

35. Delavenne E, Mounier J, Déniel F, Barbier G, Le Blay G: **Biodiversity of antifungal lactic acid bacteria isolated from raw milk samples from cow, ewe and goat over one-year period.** *Int J Food Microbiol* 2012, **155**(3)**:**185–190.

36. Medina RB, Oliszewski R, Abeijón Mukdsi MC, Van Nieuwenhove CP, González SN: **Sheep and goat's dairy products from South America: Microbiota and its metabolic activity.** *Small Ruminant Res* 2011, **101**(1–3)**:**84–91.

37. Scintu MF, Piredda G: **Typicity and biodiversity of goat and sheep milk products.** *Small Ruminant Res* 2007, **68**(1)**:**221–231.

38. Moraes PM, Perin LM, Tassinari Ortolani MB, Yamazi AK, Viçosa GN, Nero LA: **Protocols for the isolation and detection of lactic acid bacteria with bacteriocinogenic potential.** *LWT - Food Sci Technol* 2010, **43**(9)**:**1320–1324.

39. Moraes PM, Perin LM, Todorov SD, Silva A Jr, Franco BDGM, Nero LA: **Bacteriocinogenic and virulence potential of *Enterococcus* isolates obtained from raw milk and cheese.** *J Appl Microbiol* 2012, **113**(2)**:**318–328.

40. Riley MA, Wertz JE: **Bacteriocins: evolution, ecology, and application.** *Annu Rev Microbiol* 2002, **56**(1)**:**117–137.

41. Bromberg R, Moreno I, Delboni RR, Cintra HC, Oliveira PTV: **Characteristics of the bacteriocin produced by *Lactococcus lactis* subsp. *cremoris* CTC 204 and the effect of this compound on the mesophilic bacteria associated with raw beef.** *World J Microbiol Biotechnol* 2005, **21**(3)**:**351–358.

42. de Martinis ECP, Santarosa PR, Freitas FZ: **Caracterização preliminar de bacteriocinas produzidas por seis cepas de bactérias láticas isoladas de produtos cárneos embalados a vácuo.** *Cien Tecnol Alim* 2003, **23**(2)**:**195–199.

43. Lewus CB, Sun S, Montville TJ: **Production of an amylase-sensitive bacteriocin by an atypical *Leuconostoc paramesenteroides* strain.** *Appl Environ Microbiol* 1992, **58**(1)**:**143–149.

44. Todorov SD, Dicks LMT: **Effect of modified MRS medium on production and purification of antimicrobial peptide ST4SA produced by *Enterococcus mundtii*.** *Anaerobe* 2009, **15**(3)**:**65–73.

45. Campos CA, Rodríguez Ó, Calo-Mata P, Prado M, Barros-Velázquez J: **Preliminary characterization of bacteriocins from *Lactococcus lactis, Enterococcus faecium* and *Enterococcus mundtii* strains isolated from turbot (*Psetta maxima*).** *Food Res Int* 2006, **39**(3)**:**356–364.

46. Giraffa G, Neviani E: **DNA-based, culture-independent strategies for evaluating microbial communities in food-associated ecosystems.** *Int J Food Microbiol* 2001, **67**(1)**:**19–34.

47. Mohania D, Nagpal R, Kumar M, Bhardwaj A, Yadav M, Jain S, Marotta F, Singh V, Parkash O, Yadav H: **Molecular approaches for identification and characterization of lactic acid bacteria.** *J Digest Dis* 2008, **9**(4)**:**190–198.

48. Moraes PM, Perin LM, Silva A Jr, Nero LA: **Comparison of phenotypic and molecular tests to identify lactic acid bacteria.** *Braz J Microbiol* 2013, **in press:**in press. in press.

49. Alegría Á, Delgado S, Roces C, López B, Mayo B: **Bacteriocins produced by wild *Lactococcus lactis* strains isolated from traditional, starter-free cheeses made of raw milk.** *Int J Food Microbiol* 2010, **143**(1)**:**61–66.

50. Gevers D, Huys G, Swings J: **Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species.** *FEMS Microbiol Lett* 2001, **205**(1)**:**31–36.

51. Mohammed M, Abd El-Aziz H, Omran N, Anwar S, Awad S, El-Soda M: **Rep-PCR characterization and biochemical selection of lactic acid bacteria isolated from the Delta area of Egypt.** *Int J Food Microbiol* 2009, **128**(3)**:**417–423.

52. McAuliffe O, Ryan MP, Ross P, Hill C, Breeuwer P, Abee T: **Lacticin 3147, a broad-spectrum bacteriocin which selectively dissipates the membrane potential.** *Appl Environ Microbiol* 1998, **64**(2)**:**439–445.

53. Javed A, Masud T, Ul Ain Q, Imran M, Maqsood S: **Enterocins of *Enterococcus faecium*, emerging natural food preservatives.** *Ann Microbiol* 2011, **61**(4)**:**699–708.

54. Cintas LM, Casaus P, Holo H, Hernandez PE, Nes IF, Håvarstein LS: **Enterocins L50A and L50B, two novel bacteriocins from *Enterococcus faecium* L50, are related to staphylococcal hemolysins.** *J Bacteriol* 1998, **180**(8)**:**1988–1994.

55. Franz CMAP, Van Belkum MJ, Holzapfel WH, Abriouel H, Galvez A: **Diversity of enterococcal bacteriocins and their grouping in a new classification scheme.** *FEMS Microbiol Rev* 2007, **31**(3)**:**293–310.

56. Mulders JWM, Boerrigter IJ, Rollema HS, Siezen RJ, de Vos WM: **Identification and characterization of the lantibiotic nisin Z, a natural nisin variant.** *Eur J Biochem* 1991, **201**(3)**:**581–584.

CAPÍTULO 2. Virulence, antibiotic resistance and biogenic amines of bacteriocinogenic lactococci and enterococci isolated from goat milk

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Title page

Virulence, antibiotic resistance and biogenic amines of bacteriocinogenic lactococci and enterococci isolated from goat milk

Running title: Virulence of LAB isolated from goat milk

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ABSTRACT

The present study aimed to investigate the virulence, antibiotic resistance and biogenic amine production in bacteriocinogenic lactococci and enterococci isolated from goat milk in order to evaluate their safety. Twenty-nine bacteriocinogenic lactic acid bacteria (LAB: 11 *Lactococcus* spp., and 18 *Enterococcus* spp.) isolated from raw goat milk were selected and subjected to PCR to identify *gel*E, *cyl*A, *hyl*, *asa*1, *esp*, *efa*A, *ace*, *van*A, *van*B, *hdc*1, *hdc*2, *tdc* and *odc* genes. The expression of virulence factors (gelatinase, haemolysis, lipase, DNAse, tyramine, histamine, putrescine) in different incubation temperatures was assessed by phenotypic methods, as well as the resistance to vancomycin, gentamicin, chloramphenicol, ampicillin and rifampicin (using Etest®). The tested isolates presented distinct combinations of virulence related genes, but not necessarily the expression of such factors. The relevance of identifying virulence-related genes in bacteriocinogenic LAB was highlighted, demanding for care in their usage as starter cultures or biopreservatives due to the possibility of horizontal gene transfer to other bacteria in food systems.

Keywords: bacteriocins, virulence, antibiotic resistance, biogenic amines, lactic acid bacteria, goat milk.

1. Introduction

Lactic acid bacteria (LAB) constitute a microbial group naturally present in the autochthonous microbiota of animal origin foods. This group has a particular interest by food industries due to their technological properties, being often considered as starter cultures to produce fermented products (Buckenhüskes, 1993). In addition, LAB are known to be able to produce many antimicrobial substances, such as bacteriocins, being focus of scientific interest due to their ability to control foodborne pathogens and assure safety to food commodities (Castellano et al., 2008; Cotter et al., 2005).

Goat milk presents a complex lactic microbiota. This complexity is the main responsible for the particular characteristics presented by fermented dairy products produced with goat milk, leading to several studies to characterize their technological properties in order to select potential starter cultures (Badis et al., 2004; Schirru et al., 2012). Given its complexity, the autochthonous microbiota of goat milk is also considered a potential source of bacteriocinogenic LAB, being often studied to isolate and identify strains capable to produce different bacteriocins possessing different antimicrobial potential (Herreros et al., 2005; Perin & Nero, 2014; Schirru et al., 2012).

Despite the interest of using LAB as biopreservatives and starter cultures, the use of isolated strains demands caution due to their possible virulence potential. LAB, especially *Enterococcus* spp., can carry virulence genes and express them in food products, representing hazards for consumers (Moraes et al., 2012; Nieto-Arribas et al., 2011). LAB can also present resistance to distinct antibiotics and carry genes related to this characteristic, enhancing their virulence potential (Ammor et al., 2007; Levy & Marshall, 2004). Considering these characteristics, the possibility of horizontal transfer of such genes among LAB and other bacteria is a concern in the food industry (Hummel et al., 2007; Toomey et al., 2010). For instance, *Lactococccus* spp. is not usually

associated to virulence, but can carry such genes and become a virulence reservoir in a food system, and also receive genes from virulent strains (Herreros et al., 2005).

The present work aimed to investigate the virulence potential, antibiotic resistance and biogenic amine production by bacteriocinogenic LAB previously isolated from raw goat milk, using molecular and phenotypical tools.

2. Material and Methods

2.1. Microorganisms

In a previous study (Perin & Nero, 2014), a culture collection composed of 57 LAB isolates obtained from raw goat milk was characterized as bacteriocinogenic by phenotypical and molecular methods, identified as *Lactococcus* spp. (n = 24) and *Enterococcus* spp. (n = 33) by sequencing, and grouped according rep-PCR profiles. Based on these characteristics, 11 *Lactococcus* spp. (named GLc01, GLc02, GLc03: *L. lactis*; and GLc05, GLc13, GLc17, GLc18, GLc20, GLc22, GLc23, GLc24: *L. lactis* subsp. *lactis*) and 18 *Enterococcus* spp. (GEn01, GEn02, GEn03, GEn09, GEn16: *E. durans*; GEn18, GEn19, GEn20, GEn22, GEn23, GEn25: *E. faecalis*; GEn26, GEn 27, GEn28, GEn30, GEn31, GEn32: *E. faecium*; GEn33: *E. hirae*) were selected for the present study. The isolates were stored in de Man, Rogosa and Sharpe (MRS) broth (Oxoid Ltd., Basingstoke, England) supplemented with 25 % (v/v) glycerol at -80 °C. For use, stock cultures were streaked on MRS agar (Oxoid), incubated at 35 °C for 24 h, and then isolated colonies were transferred to MRS broth (Oxoid) and incubated at 35 °C for 24 h.

The DNA for genes encoding virulence, antibiotic resistance and amino acid decarboxylase was extracted using ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA). Then, the DNA concentration was estimated using NanoDrop2000

(Thermo Scientific Inc., Waltham, MA, USA). The presence of the following virulence, antibiotic resistance and biogenic amine related genes were investigated: *gel*E (gelatinase), *cyl*A (cytolisin), *hyl* (hyaluronidase), *asa*1 (aggregation substance), *esp* (enterococcal surface protein), *efa*A (endocarditis antigen), *ace* (adhesion of collagen), *van*A and *van*B (both related to vancomycin resistance), and genes for amino acid decarboxylases: *hdc*1 and *hdc*2 (both related to histidine decarboxylase), *tdc* (tyrosine decarboxylase), and *odc* (ornithine decarboxylase). The genes were detected using PCR conditions described by Vankerckhoven et al. (2004), Rivas et al. (2005), and Martín-Platero et al. (2009). PCR products were electrophoresed in agarose gels from 0.8 to 2.0% (w/v) in 0.5 × TBE and stained with TBE buffer containing 0.5 μg/mL ethidium bromide (Sigma-Aldrich, Inc., St. Louis, MO, USA). Primers, annealing temperatures and fragment sizes are described in Table 1.

Table 1: Primers sequences utilized in the investigation of genes for virulence and biogenic amines.

target gene*	Primers	annealing	fragment size	Reference
*gel*E	TATGACAATGCTTTTTGGGAT	47°C	213 bp	Vankerckhoven et al., 2004
	AGATGCACCCGAAATAATATA			
*cyl*A	ACTCGGGGATTGATAGGC	52°C	688 bp	Vankerckhoven et al., 2004
	GCTGCTAAAGCTGCGCTT			
hyl	ACAGAAGAGCTGCAGGAAATG	53°C	276 bp	Vankerckhoven et al., 2004
	GACTGACGTCCAAGTTTCCAA			
*asa*1	GCACGCTATTACGAACTATGA	50°C	375 bp	Vankerckhoven et al., 2004
	TAAGAAAGAACATCACCACGA			
esp	AGATTTCATCTTTGATTCTTG	47°C	510 bp	Vankerckhoven et al., 2004
	AATTGATTCTTTAGCATCTGG			
*efa*A	GCCAATTGGGACAGACCCTC	57°C	688 bp	Martin-Platero et al., 2009
	CGCCTTCTGTTCCTTCTTTGGC			
ace	GAATTGAGCAAAAGTTCAATCG	48°C	1008 bp	Martin-Platero et al., 2009
	GTCTGTCTTTTCACTTGTTTC			
*van*A	TCTGCAATAGAGATAGCCGC	52°C	377 bp	Martin-Platero et al., 2009
	GGAGTAGCTATCCCAGCATT			
*van*B	GCTCCGCAGCCTGCATGGACA	60°C	529 bp	Martin-Platero et al., 2009
	ACGATGCCGCCATCCTCCTGC			
*hdc*1	AGATGGTATTGTTTCTTATG	46°C	367 bp	Rivas et al., 2005
	AGACCATACACCATAACCTT			
*hdc*2	AAYTCNTTYGAYTTYGARAARGARG	50°C	534 bp	Rivas et al., 2005
	ATNGGNGANCCDATCATYTTRTGNCC			
tdc	GAYATNATNGGNATNGGNYTNGAYCARG	55°C	924 bp	Rivas et al., 2005
	CCRTARTCNGGNATAGCRAARTCNGTRTG			
odc	GTNTTYAAYGCNGAYAARCANTAYTTYGT	54°C	1446 bp	Rivas t al., 2005
	ATNGARTTNAGTTCRCAYTTYTCNGG			

* *gel*E (gelatinase), *cyl*A (cytolisin), *hyl* (hyaluronidase), *asa*1 (aggregation substance), *esp* (enterococcal surface protein), *efa*A (endocarditis antigen), *ace* (adhesion of collagen), *van*A and *van*B (vancomycin resistance), *hdc*1 and *hdc*2 (histidine decarboxylase), *tdc* (tyrosine decarboxylase), and *odc* (ornithine decarboxylase)

2.2. Virulence activity

Cultures of the 29 selected isolates were subjected to phenotypical tests to identify their virulence activity, according Barbosa et al. (2010). All tests were performed with different combinations of time and incubation temperature in order to verify the production of the virulence factors in diverse conditions, detailed in the following, in three independent trials.

Gelatinase production was verified by spotting 1 µL aliquots of the 24 h cultures onto the surface of Luria Bertani agar (LB; Becton, Dickinson and Company - BD, Franklin Lakes, NJ, USA) supplemented with 3% (w/v) gelatin (BD) and incubated at 37 °C and 42 °C for 48 h, 25 °C for 72 h, and 10 °C and 15 °C for 10 days. After incubation, the plates were maintained at 4 °C for 4 h and the hydrolysis of gelatin was recorded by the formation of opaque halos around the colonies.

Hemolytic activity was assessed by streaking the cultures onto trypticase soy agar (Oxoid) supplemented with defibrinated horse blood at 5% (v/v) and incubated at 37 °C and 42 °C for 24 h, 25 °C for 48 h, and 10 °C and 15 °C for 10 days. The hemolysis formed by each isolate was classified as total or β-hemolysis (clear halos around the colonies), partial or α-hemolysis (greenish halos around the colonies), and absent or γ-hemolysis.

Lipase production was assessed by spotting 1 µL of cultures onto LB plates (BD) supplemented with $CaCl_2$ (Sigma-Aldrich, at 0.2%, w/v) and Tween 80 (Sigma-Aldrich, at 1%, v/v) and incubated at 37 °C and 42 °C for 48 h, 25 °C for 72 h, and 10 °C and 15 °C for 10 days. The formation of clear halos around the colonies was recorded as lipase production.

DNAse was identified by spotting 1 µL aliquots of the cultures onto the surface of DNAse methyl green agar (BD), and incubated at 37 °C and 42 °C for 48 h,

25 °C for 72 h, and 10 °C and 15 °C for 10 days. Positive results were identified by the formation of clear halos around the colonies.

2.3. Antibiotic resistance

Cultures of the 29 selected isolates described as above were subjected to phenotypical analysis of antibiotic resistance using Etest® strips (BioMérieux SA, Marcy l'Etoile, France) for the following antibiotics: vancomycin, gentamicin, chloramphenicol, ampicillin and rifampicin. The cultures were diluted using NaCl 0.85 % (w/v) until turbidity similar to 0.5 MacFarland scale was reached, and homogeneously swabbed onto the surface of MRS (Oxoid) plates. Then, Etest® stripes were placed onto the plated surface, and the plates were incubated at 37 °C for 24 h. Considering the halo formation around the strips, the minimum inhibitory concentrations (MIC) were estimated (µg/mL) for each antibiotic against each strain, which was classified as presenting resistance (R), intermediary resistance (IR), or sensitivity (S), according instructions of the manufacturer for rifampicin, and the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2014) for other antibiotics tested.

2.4. Biogenic amines production

The production of biogenic amines by the selected LAB isolates was evaluated according to Bover-Cid & Holzapfel (1999). Decarboxylase production was induced by five consecutive transfers of 0.5 ml aliquots of the cultures into MRS broth (Oxoid) supplemented with pyridoxal-5-phosphate at 0.005% (w/v, Sigma-Aldrich) and with each one of the biogenic amine precursors at 0.1% (w/v): tyrosine free base (for tyramine), histidine monohydrochloride (for histamine), and ornithine monohydrochloride (for putrescine) (all from Sigma Aldrich). Each culture was

incubated at 37 °C for 24 h, and the end cultures were streaked onto decarboxylase agar, a MRS agar modified according to Joosten & Northolt (1989), supplemented with one of each biogenic amine precursor described as above, at 1 % (w/v). The plates were incubated at 37 °C and 42 °C for 4 days, 25 °C for 7 days, and 10 °C and 15 °C for 14 days, and positive results were recorded by color changing from yellow to purple.

3. Results and Discussion

Table 2 shows the results for virulence genes and virulence activity of the tested LAB isolates. Only four isolates (*L. lactis* GLc03, *L. lactis* subsp. *lactis* GLc13, *E. durans* GEn02 and *E. durans* GEn03) from the tested 29 LAB did not present any virulence genes. A high frequency of positive results were observed for *gel*E in *Lactococcus* spp. (six strains) and *Enterococcus* spp. (fourteen strains); however, only three of them presented production of gelatinase by phenotypic testing at 25, 37 and 42 °C, being all *E. faecalis* (GEn20, GEn22, GEn23). All *gel*E positive *Lactococcus* spp. did not produce gelatinase (Table 2). Eaton & Gasson (2001) described that *gel*E expression is highly influenced by the culture conditions, and the laboratory manipulation of the strains can result in the loss of the structural genes, and can explain the loss of gelatinase activity during *in vitro* tests. According to Lopes et al. (2006), the presence of *gel*E gene is probably not enough for gelatinase activity, since the complete *fsr* operon seems to be essential for its expression. However, the *fsr* operon seems to be easily damaged, lost or suffer from deletions, mainly during the freezing of the cells in the laboratory. The detection of *gel*E gene in other *Enterococcus* species, other than *E. faecalis* and *E. durans*, was already identified by Lopes et al. (2006) and Moraes et al. (2012), and the present study could be the first to identify *Lactococcus* strains positive for the *gel*E gene. However, further studies must be conducted for searching the presence of the *fsr* operon in these strains.

Table 2. Results for virulence genes and virulence activity of selected bacteriocinogenic LAB obtained from raw goat milk.

species	isolate	genes*							hemolysis activity**					gelatinase production***				
		*asa*1	*cyl*A	*efa*A	*esp*	*gel*E	*hyl*	*ace*	10 °C, 10d	15 °C, 10d	25 °C, 48h	37 °C, 24h	42 °C, 24h	10 °C, 10d	15 °C, 10d	25 °C, 72h	37 °C, 48h	42 °C, 48h
E. durans	GEn01	-	-	-	-	+	-	-	γ	γ	γ	γ	γ	-	-	-	-	-
	GEn02	-	-	-	-	-	-	-	γ	γ	γ	γ	γ	-	-	-	-	-
	GEn03	-	-	-	-	-	-	-	γ	γ	γ	γ	γ	-	-	-	-	-
	GEn09	+	-	+	+	-	-	-	γ	γ	γ	γ	γ	-	-	-	-	-
	GEn16	+	-	-	+	-	-	-	γ	α	α	α	α	-	-	-	-	-
E. faecalis	GEn18	-	-	-	+	+	-	-	γ	γ	γ	γ	γ	-	-	-	-	-
	GEn19	+	-	+	+	+	-	+	γ	α	α	α	α	-	-	-	-	-
	GEn20	+	-	+	+	+	-	+	γ	γ	γ	γ	γ	-	-	+	+	+
	GEn22	+	-	+	+	+	-	+	γ	γ	γ	γ	γ	-	-	+	+	+
	GEn23	+	-	+	+	+	-	+	γ	γ	γ	γ	γ	-	-	+	+	+
	GEn25	+	-	+	+	+	-	+	γ	α	γ	γ	γ	-	-	-	-	-
E. faecium	GEn26	+	-	-	+	+	-	-	γ	α	α	α	α	-	-	-	-	-
	GEn27	+	+	+	+	+	-	-	γ	α	α	α	α	-	-	-	-	-
	GEn28	+	-	-	+	+	-	+	γ	α	α	α	α	-	-	-	-	-
	GEn30	+	-	+	+	+	-	-	γ	α	α	α	α	-	-	-	-	-
	GEn31	+	-	+	+	+	-	+	γ	γ	γ	γ	γ	-	-	-	-	-
	GEn32	+	-	+	+	+	-	-	γ	α	α	α	α	-	-	-	-	-
E. hirae	GEn33	+	-	+	+	+	-	+	γ	α	α	α	γ	-	-	-	-	-
L. lactis	GLc01	+	-	+	+	+	-	-	γ	α	α	α	α	-	-	-	-	-
	GLc02	+	-	-	+	-	-	-	γ	γ	γ	γ	γ	-	-	-	-	-
	GLc03	-	-	-	-	-	-	-	γ	γ	γ	γ	γ	-	-	-	-	-

Obs.: None isolate presented positive results for lipases and DNAse production after incubation at 37 °C and 42 °C for 48h, 25 °C for 72h, and 10 °C and 15 °C for 10 days.

* *gel*E: gelatinase; *cyl*A: cytolysin; *hyl*: hyaluronidase; *asa*1: aggregation substance; *esp*: enterococcal surface protein; *efa*A: endocarditis antigen; *ace*: adhesion of collagen. +: presence; -: absence. ** α: partial hemolysis, γ: absence of hemolysis. *** +: positive; -: negative.

Complement of Table 2.

Species	isolate	genes*							hemolysis activity**					gelatinase production***				
		*asa*1	*cyl*A	*efa*A	*esp*	*gel*E	*hyl*	*ace*	10 °C, 10d	15 °C, 10d	25 °C, 48h	37 °C, 24h	42 °C, 24h	10 °C, 10d	15 °C, 10d	25 °C, 72h	37 °C, 48h	42 °C, 48h
L. lactis subsp. *lactis*	GLc05	-	-	+	+	-	-	-	γ	γ	γ	γ	γ	-	-	-	-	-
	GLc13	-	-	-	-	-	-	-	γ	γ	γ	γ	γ	-	-	-	-	-
	GLc17	-	-	-	+	+	-	-	γ	γ	γ	γ	γ	-	-	-	-	-
	GLc18	-	-	-	-	-	-	+	γ	γ	γ	γ	γ	-	-	-	-	-
	GLc20	+	-	+	+	+	-	-	γ	γ	γ	γ	γ	-	-	-	-	-
	GLc22	-	-	-	+	+	-	+	γ	α	γ	γ	γ	-	-	-	-	-
	GLc23	+	-	-	+	+	-	-	γ	γ	γ	γ	γ	-	-	-	-	-
	GLc24	-	-	-	+	+	-	-	γ	α	γ	α	α	-	-	-	-	-

Obs.: None isolate presented positive results for lipases and DNAse production after incubation at 37 °C and 42 °C for 48h, 25 °C for 72h, and 10 °C and 15 °C for 10 days.

* *gel*E: gelatinase; *cyl*A: cytolysin; *hyl*: hyaluronidase; *asa*1: aggregation substance; *esp*: enterococcal surface protein; *efa*A: endocarditis antigen; *ace*: adhesion of collagen. +: presence; -: absence. ** α: partial hemolysis, γ: absence of hemolysis. *** +: positive; -: negative.

Only one *E. faecium* isolate (GEn27) presented a positive result for *cyl*A, but α-hemolysis was observed by phenotypical testing in twelve isolates, including GEn27 (Table 2). Mostly probably, other lytic genes are involved in this hemolysis reaction. Considering the low temperatures of incubation, no isolates presented hemolytic activity at 10 °C and two isolates (GEn25 and GLc22) were active only at 15 °C. In general, the other positive isolates were active from 15 °C to 42 °C of incubation (Table 2). The high level of the cytolysin operon expression is reached by the generation of quorum sensing; considering that the growth of the strains is slower at low temperatures, i.e. 10 °C, the autoinduction by quorum sensing may not have occurred (Van Tyne et al., 2013).

No isolates presented β-hemolysis. Cytolysin is a substance with ambiguous activity: it is virulent due to its hemolytic potential, but it is also considered as an antibacterial/bacteriocin according to Cotter et al. (2005) classification. Its molecular structure contains lanthionine residues, which determine the classification of cytolysin as a class I bacteriocin (Haas et al., 2002). The transference of the operon encoding cytolysin between strains can occur since this operon is located on transmissible plasmids and a mobile pathogenicity island (Van Tyne et al., 2013). The expression of cytolysin requires the presence and functionality of eight genes: *cyl*L_L and *cyl*L_S, which encode the cytolysin structural peptides, *cyl*M, *cyl*B, *cyl*A, and *cyl*I, which encode proteins enrolled in the post-translational modifications into peptides (secretion, extracellular activation, and immunity) and *cyl*R1 and *cyl*R2, which are regulators of cytolysin expression (Cox et al., 2005). Another aspect to be considered is that the cytolysin operon could be transcribed at low levels in cases of an uninduced state, as in the case of *in vitro* tests. Van Tyne et al. (2013) already described the cytolysin transcription as a complex system highly influenced by the environmental conditions of culturing. *E. faecalis* and *E. faecium* isolated from foods were already described as

capable of producing cytolysin, despite this characteristic being more common in isolates obtained from clinical samples (Eaton & Gasson, 2001; Semedo et al., 2003).

High frequencies of positive results were observed for *asa*1, *esp*, *efa*A, and *ace* in *Lactococcus* and *Enterococcus* isolates (Table 2). These genes are related to the production of different substances enrolled in the microbial colonization and adhesion at biotic and non-biotic surfaces, and also related to evasion of the host immune system (Shankar et al., 1999; Valenzuela et al., 2009). Gomes et al. (2008) identified high frequencies of *efa*A positive enterococci, and demonstrated the relevance of this gene in the persistence of this genus in a variety of environments.

No tested isolates presented a positive result for *hyl*, which is related to the production of hyaluronidase (Table 2). This substance is related to the dispersion of the producing bacteria or their toxins in the host tissue, and can also interact with lymphocyte receptors inducing immune diseases (Girish & Kemparaju, 2007; Sousa, 2003). No isolates presented positive results for lipase and DNAse activity on phenotypical tests, independently of the incubation condition. These virulence factors are usually researched in clinical isolates, being detected at high frequencies in *Enterococcus* spp., and frequently associated to competitive advantages of pathogenic strains (Semedo et al., 2003).

The diversity patterns of positive results for virulence genes and virulence activity was already described in LAB isolated from foods, especially *Enterococcus* spp. (Majhenič et al., 2005; Moraes et al., 2012; Valenzuela et al., 2009). *Lactococcus* spp. can also carry distinct virulence genes, but their presence in the genome is not a proper indication of the pathogenicity of this genus; the obtained data indicate their poor capability of expressing such genes (Table 1), as observed in similar studies (Casalta & Montel, 2008; Fernández et al., 2010).

Table 3. Results for the antibiotic resistance related genes and minimum inhibitory concentration (MIC) of selected bacteriocinogenic LAB obtained from raw goat milk.

Species	isolate	genes*		MIC (µg/mL)**				
		Vana	*van*B	VAN	GEN	CHL	AMP	RIF
E. durans	GEn01	-	-	0.25 (S)	8.0 (S)	3.0 (S)	0.94 (S)	0.004 (S)
	GEn02	-	-	0.50 (S)	8.0 (S)	0.75 (S)	0.64 (S)	0.008 (S)
	GEn03	-	-	0.38 (S)	6.0 (S)	1.5 (S)	6.0 (S)	0.003 (S)
	GEn09	-	-	1.0 (S)	12.0 (S)	2.0 (S)	0.75 (S)	0.006 (S)
	GEn16	-	-	1.0 (S)	12.0 (S)	2.0 (S)	0.64 (S)	0.004 (S)
E. faecalis	GEn18	-	-	1.0 (S)	AH (R)	2.0 (S)	0.25 (S)	0.25 (S)
	GEn19	-	-	1.5 (S)	AH (R)	1.0 (S)	0.125 (S)	3.0 (IR)
	GEn20	+	-	3.0 (S)	128.0 (S)	1.5 (S)	0.25 (S)	2.0 (IR)
	GEn22	+	-	3.0 (S)	64.0 (S)	2.0 (S)	0.38 (S)	0.75 (S)
	GEn23	+	-	1.5 (S)	128.0 (S)	1.0 (S)	0.25 (S)	1.0 (S)
	GEn25	-	-	1.5 (S)	AH (R)	2.0 (S)	0.19 (S)	0.19 (S)
E. faecium	GEn26	-	-	1.5 (S)	48.0 (S)	2.0 (S)	0.25 (S)	3.0 (IR)
	GEn27	-	-	AH (R)	64.0 (S)	2.0 (S)	0.75 (S)	2.0 (IR)
	GEn28	-	-	1.5 (S)	64.0 (S)	1.5 (S)	0.38 (S)	3.0 (IR)
	GEn30	-	-	1.5 (S)	64.0 (S)	1.0 (S)	0.19 (S)	2.0 (IR)
	GEn31	-	-	1.0 (S)	48.0 (S)	1.5 (S)	0.19 (S)	2.0 (IR)
	GEn32	-	-	1.0 (S)	48.0 (S)	1.5 (S)	0.25 (S)	3.0 (IR)
E. hirae	GEn33	+	-	1.5 (S)	12.0 (S)	2.0 (S)	0.25 (S)	AH (R)
L. lactis	GLc01	-	-	0.38 (S)	24.0 (S)	1.5 (S)	0.50 (S)	1.5 (IR)
	GLc02	-	-	0.50 (S)	12.0 (S)	2.0 (S)	0.125 (S)	0.006 (S)
	GLc03	-	-	0.50 (S)	8.0 (S)	4.0 (S)	0.25 (S)	0.004 (S)
L. lactis subsp. *lactis*	GLc05	-	-	0.38 (S)	24.0 (S)	1.5 (S)	0.50 (S)	0.004 (S)
	GLc13	-	-	0.38 (S)	128.0 (S)	8.0 (S)	0.125 (S)	AH (R)
	GLc17	-	-	1.5 (S)	192.0 (R)	2.0 (S)	0.64 (S)	12.0 (R)
	GLc18	-	-	0.75 (S)	32.0 (S)	1.5 (S)	0.25 (S)	0.5 (S)
	GLc20	+	-	0.38 (S)	AH (R)	2.0 (S)	0.19 (S)	6.0 (R)
	GLc22	-	-	1.0 (S)	AH (R)	2.0 (S)	0.25 (S)	0.25 (S)
	GLc23	+	-	1.0 (S)	128.0 (S)	2.0 (S)	0.94 (S)	AH (R)
	GLc24	-	-	AH (R)	48.0 (S)	1.0 (S)	0.19 (S)	2.0 (IR)

* *van*A and *van*B: vancomycin resistance related genes. +: presence; -: absence. ** VAN: vancomycin; GEN: gentamicin; CHL: chloramphenicol; AMP: ampicillin; RIF: rifampicin. R: resistant; S: sensitive; IR: intermediary resistance; AH: absence of inhibition zone. Breaking points have been determined according to manufacturer instructions (bioMeriéux, France), and (EUCAST, 2014).

Table 3 presents the results for vancomycin resistance genes, and the MIC values for each tested antibiotic and each isolate. No isolate presented a positive result for *van*B. Conversely, four *Enterococcus* (GEn20, GEn22, GEn23, GEn33), and two *Lactococcus* (GLc20, GLc23) presented positive results for *van*A, but absence of

resistance to vancomycin by the phenotypical test. According Ribeiro et al. (2007), the resistance to vancomycin by clinical and food (dairy) isolates occurs due to the transfer of *van*A by genetic elements, such as transposons, and not by selection of resistant strains. Vancomycin resistant *Enterococcus* are considered a frequent cause of hospital infections, therefore their characterization and control of possible sources, such as foods, is necessary (Mathur & Singh, 2005). Only two isolates presented the resistance phenotype to vancomycin (GLc24 and GEn27), and both were negative for *van*A and *van*B, indicating that this characteristic could be related to other resistance types, such as VanC, VanD, VanE or VanG (Courvalin, 2006). Isolates that presented *van*A did not have a resistance profile to vancomycin, indicating absence of gene expression as observed previously by Khan et al. (2005). It is important to underline that *van*A and *van*B are normally associated to a location on plasmid DNA, however *van*C, *van*D, *van*E and *van*G are located on bacterial chromosome (Martín-Platero et al., 2009). Based on these locations, *van*A and *van*B are major concerns for the safety of spreading antibiotic resistance via horizontal gene transfer.

According to EUCAST (2014), strains with a MIC value higher than 32 µg/mL are considered resistant to gentamicin. Three *Lactococcus* and three *Enterococcus* presented resistance to gentamicin as detected by Etest® (Table 3). According to EUCAST (2014), strains that present a MIC value higher than 128 µg /mL have acquired resistance mechanisms and can be reported as high-level aminoglycoside resistant, which was the case for the *Lactococcus* spp. GLc17. Enterococci are usually described as having intrinsic resistance to aminoglycosides, such as gentamicin, and high levels of resistance were already described in strains obtained from dairy products (Donabedian et al., 2003; Mathur & Singh, 2005). In contrast, *Lactococcus* isolates have been described as sensitive or resistant to gentamicin (Ammor et al., 2007; Florez et al., 2005; Mathur & Singh, 2005). All tested isolates presented sensitivity to

chloramphenicol and ampicillin (Table 3). Similar results were observed by Toomey et al. (2010) in *Lactococcus* and *Enterococcus* isolates obtained from meat products. *Lactococcus* is usually described as sensitive to β-lactam antibiotics, such as penicillin and ampicillin, but resistant strains were already identified from culture collections obtained from foods of animal origin (Ammor et al., 2007; Perreten et al., 1997).

Rifampicin is a broad-spectrum antibiotic used in the control of *Mycobacterium tuberculosis*, and is usually active against *Lactococcus* (Ammor et al., 2007). Five isolates (four *Lactococcus* and one *Enterococcus*) presented resistance to this antibiotic, and ten isolates (two *Lactococcus* and eight *Enterococcus*) presented intermediate resistance (Table 3). The resistance to rifampicin by *Lactococcus* was described by Van Veen & Konings (1998) as a consequence of the multidrug transport mechanisms of this genus, which presented homology to the same mechanism described for humans.

There is a current concern in identifying LAB isolates from foods that present antibiotic resistance genes, because they can act as reservoirs and also transfer such genes to pathogenic bacteria (Valenzuela et al., 2009). However, related studies are more focused on investigating the virulence factors of *E. faecium* and *E. faecalis* species, due to their usual association to pathogenicity, while studies on *Lactococcus* and *Lactobacillus* are limited to a few (Mathur & Singh, 2005). The obtained results indicate a generally low frequency of positive results for vancomycin related genes, and also low frequencies of chloramphenicol and ampicillin resistant isolates, but high frequency of isolates resistant to rifampicin.

Table 4. Results for the biogenic amines related genes and tyrosine decarboxylase production by selected bacteriocinogenic LAB obtained from raw goat milk

Species	isolate	genes*				tyramine**				
		*hdc*1	*hdc*2	*tdc*	*odc*	10 °C, 10d	15 °C, 10d	25 °C, 48h	37 °C, 24h	42 °C, 24h
E. durans	GEn01	-	-	+	-	-	-	-	+	+
	GEn02	-	-	-	-	-	-	-	+	+
	GEn03	-	-	+	-	-	-	+	+	+
	GEn09	-	-	+	-	-	-	-	-	-
	GEn16	-	-	+	-	-	-	+	+	+
E. faecalis	GEn18	-	-	-	-	-	+	+	+	+
	GEn19	-	-	+	-	-	-	-	-	-
	GEn20	-	+	+	-	-	+	+	+	+
	GEn22	-	+	+	-	-	+	+	+	+
	GEn23	-	+	+	-	-	+	+	+	+
	GEn25	-	-	+	-	-	+	+	+	+
E. faecium	GEn26	-	-	+	-	-	-	-	-	-
	GEn27	-	-	+	-	-	-	-	-	+
	GEn28	-	-	+	-	-	-	-	-	-
	GEn30	-	-	+	-	-	-	-	-	-
	GEn31	-	-	+	-	-	-	-	-	-
	GEn32	-	-	+	-	-	-	-	-	-
E. hirae	GEn33	-	-	+	-	-	+	+	+	+
L. lactis	GLc01	-	-	+	-	-	-	+	+	+
	GLc02	-	-	+	-	-	-	+	+	+
	GLc03	-	-	+	-	-	-	+	+	+
L. lactis subsp. *lactis*	GLc05	-	-	-	-	-	-	-	-	-
	GLc13	-	-	+	-	-	-	-	-	-
	GLc17	-	-	-	-	-	-	-	-	-
	GLc18	-	-	+	-	-	-	-	-	-
	GLc20	-	-	+	-	-	-	-	-	-
	GLc22	-	-	-	-	-	+	+	+	+
	GLc23	-	-	-	-	-	-	-	-	-
	GLc24	-	-	+	-	-	-	-	-	-

Obs.: None isolate presented positive results for histamine and putrescine production after incubation at 37 °C and 42 °C for 48h, 25 °C for 72h, and 10 °C and 15 °C for 10 days.
* *hdc*1 and *hdc*2: both related to histidine decarboxylase; *tdc*: tyrosine decarboxylase; *odc*: ornithine decarboxylase. +: presence; -: absence. ** +: positive; -: negative.

Table 4 presents the results for the presence of biogenic amines related genes and production of these substances by the tested isolates. Only three isolates identified as *E. faecalis* were positive for *hdc*2. No isolates presented the related genes *hdc*1 and *odc* and none had the capability of producing histamine and putrescine. Tyramine

related gene (*tdc*) was identified in seven *Lactococcus* spp. and sixteen *Enterococcus* spp., and it was produced in at least one incubation condition tested by fifteen isolates, most being capable of producing tyramine at 25 to 42 °C. As the biogenic amine production requires optimal growth conditions, the tested cultures were not able to promote decarboxylase synthesis and activity at lower tested temperatures, such as 10 and 15 °C. Histamine and tyramine are the biogenic amines most studied in fermented foods and starter cultures, due to their toxic effects caused by their vasoactive and psychoactive properties (Bover-Cid & Holzapfel, 1999). Despite not being identified by genetic and phenotypic tests, putrescine (encoded by *odc*) is a relevant biogenic amine due to its capability of enhancing the toxic effects of histamine and tyramine. LAB are able to convert amino acids in biogenic amines via decarboxylase or deaminase during the fermentation process, jeopardizing the quality and safety of fermented foods, which highlights the relevance of identifying the potential of producing such substances (Coton et al., 2010). The occurrence of high frequencies of *Enterococcus* capable of producing biogenic amines was already described in previous studies (Giraffa et al., 1995; Moraes et al., 2012; Valenzuela et al., 2009). It is important to highlight the occurrence of *Lactococcus* isolates possessing *tdc* and capable of producing tyramine (Table 4), as previously identified by Fernández et al. (2004). The identification of biogenic amine related genes is indicative of the toxic potential of LAB, but the intoxication by such substances demands the consumption of specific quantities (50 to 100 mg) by susceptible consumers (Santos, 1996). These results highlight the necessity of additional tests that estimate the amount of biogenic amine production by LAB isolates that are good candidates as biopreservatives in foods.

The obtained data demonstrated that bacteriocinogenic LAB isolated from raw goat milk in Minas Gerais state, Brazil, can present virulence, antibiotic resistance and biogenic amine related genes, and also express such factors. The identification of LAB

strains possessing such genes is relevant due to the possibility of horizontal transfer to other bacteria that may be able to express them. These characteristics must be properly assessed during evaluation of LAB strains to be considered as starter or biopreservative cultures. However, these tests can be used in selection of virulence free microorganisms for the future application as functional starter cultures in preparation of fermented milk products.

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References

Ammor, M.S., Flórez, A.B., Mayo, B. 2007. Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. Food Microbiology 24, 559-570.

Badis, A., Guetarni, D., Moussa Boudjema, B., Henni, D., Kihal, M. 2004. Identification and technological properties of lactic acid bacteria isolated from raw goat milk of four Algerian races. Food Microbiology 21, 579-588.

Barbosa, J., Gibbs, P.A., Teixeira, P. 2010. Virulence factors among enterococci isolated from traditional fermented meat products produced in the North of Portugal. Food Control 21, 651-656.

Bover-Cid, S., Holzapfel, W.H. 1999. Improved screening procedure for biogenic amine production by lactic acid bacteria. International Journal of Food Microbiology 53, 33-41.

Buckenhüskes, H.J. 1993. Selection criteria for lactic acid bacteria to be used as starter cultures for various food commodities. FEMS Microbiology Reviews 12, 253-271.

Casalta, E., Montel, M.-C. 2008. Safety assessment of dairy microorganisms: The *Lactococcus* genus. International Journal of Food Microbiology 126, 271-273.

Castellano, P., Belfiore, C., Fadda, S., Vignolo, G. 2008. A review of bacteriocinogenic lactic acid bacteria used as bioprotective cultures in fresh meat produced in Argentina. Meat Science 79, 483-499.

Coton, M., Romano, A., Spano, G., Ziegler, K., Vetrana, C., Desmarais, C., Lonvaud-Funel, A., Lucas, P., Coton, E. 2010. Occurrence of biogenic amine-forming lactic acid bacteria in wine and cider. Food Microbiology 27, 1078-1085.

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

Courvalin, P. 2006. Vancomycin resistance in Gram-positive cocci. Clinical Infectious Diseases 42, S25-S34.

Cox, C.R., Coburn, P.S., Gilmore, M.S. 2005. Enterococcal cytolysin: a novel two component peptide system that serves as a bacterial defense against eukaryotic and prokaryotic cells. Current Protein and Peptide Science 6, 77-84.

Donabedian, S.M., Thal, L.A., Hershberger, E., Perri, M.B., Chow, J.W., Bartlett, P., Jones, R., Joyce, K., Rossiter, S., Gay, K. 2003. Molecular characterization of gentamicin-resistant enterococci in the United States: evidence of spread from animals to humans through food. Journal of Clinical Microbiology 41, 1109-1113.

Eaton, T.J., Gasson, M.J. 2001. Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. Applied and Environmental Microbiology 67, 1628-1635.

EUCAST. 2014. Clinical breakpoints. European Committee on Antimicrobial Susceptibility Testing, Växjö, Sweden.

Fernández, E., Alegría, Á., Delgado, S., Mayo, B. 2010. Phenotypic, genetic and technological characterization of *Lactococcus garvieae* strains isolated from a raw milk cheese. International Dairy Journal 20, 142-148.

Fernández, M., Linares, D.M., Alvarez, M.A. 2004. Sequencing of the tyrosine decarboxylase cluster of *Lactococcus lactis* IPLA 655 and the development of a PCR method for detecting tyrosine decarboxylating lactic acid bacteria. Journal of Food Protection 67, 2521-2529.

Florez, A.B., Delgado, S., Mayo, B. 2005. Antimicrobial susceptibility of lactic acid bacteria isolated from a cheese environment. Canadian Journal of Microbiology 51, 51-58.

Giraffa, G., Pepe, G., Locci, F., Neviani, E., Carminati, D. 1995. Hemolytic activity, production of thermonuclease and biogenic amines by dairy enterococci. Italian Journal of Food Science 7, 341-349.

Girish, K.S., Kemparaju, K. 2007. The magic glue hyaluronan and its eraser hyaluronidase: a biological overview. Life Sciences 80, 1921-1943.

Gomes, B.C., Esteves, C.T., Palazzo, I.C.V., Darini, A.L.C., Felis, G.E., Sechi, L.A., Franco, B.D.G.M., de Martinis, E.C.P. 2008. Prevalence and characterization of *Enterococcus* spp. isolated from Brazilian foods. Food Microbiology 25, 668-675.

Haas, W., Shepard, B.D., Gilmore, M.S. 2002. Two-component regulator of *Enterococcus faecalis* cytolysin responds to quorum-sensing autoinduction. Nature 415, 84-87.

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

Hummel, A., Holzapfel, W.H., Franz, C.M.A.P. 2007. Characterisation and transfer of antibiotic resistance genes from enterococci isolated from food. Systematic and Applied Microbiology 30, 1-7.

Joosten, H.M.L.J., Northolt, M.D. 1989. Detection, growth, and amine-producing capacity of lactobacilli in cheese. Applied and Environmental Microbiology 55, 2356-2359.

Khan, S.A., Nawaz, M.S., Khan, A.A., Hopper, S.L., Jones, R.A., Cerniglia, C.E. 2005. Molecular characterization of multidrug-resistant *Enterococcus* spp. from poultry and dairy farms: detection of virulence and vancomycin resistance gene markers by PCR. Molecular and Cellular Probes 19, 27-34.

Levy, S.B., Marshall, B. 2004. Antibacterial resistance worldwide: causes, challenges and responses. Nature Medicine 10, S122-S129.

Lopes, M.F.S., Simões, A.P., Terneiro, R., Marques, J.J.F., Crespo, M.T.B. 2006. Activity and expression of a virulence factor, gelatinase, in dairy enterococci. International Journal of Food Microbiology 112, 208-214.

Majhenič, A.Č., Rogelj, I., Perko, B. 2005. Enterococci from Tolminc cheese: population structure, antibiotic susceptibility and incidence of virulence determinants. International Journal of Food Microbiology 102, 239-244.

Martín-Platero, A.M., Valdivia, E., Maqueda, M., Martínez-Bueno, M. 2009. Characterization and safety evaluation of enterococci isolated from Spanish goats' milk cheeses. International Journal of Food Microbiology 132, 24-32.

Mathur, S., Singh, R. 2005. Antibiotic resistance in food lactic acid bacteria - a review. International Journal of Food Microbiology 105, 281-295.

Moraes, P.M., Perin, L.M., Todorov, S.D., Silva, A., Jr., Franco, B.D.G.M., Nero, L.A. 2012. Bacteriocinogenic and virulence potential of *Enterococcus* isolates obtained from raw milk and cheese. Journal of Applied Microbiology 113, 318-328.

Nieto-Arribas, P., Seseña, S., Poveda, J.M., Chicón, R., Cabezas, L., Palop, L. 2011. *Enterococcus* populations in artisanal Manchego cheese: Biodiversity, technological and safety aspects. Food Microbiology 28, 891-899.

Perin, L.M., Nero, L.A. 2014. Antagonistic lactic acid bacteria isolated from goat milk and identification of a novel nisin variant *Lactococcus lactis*. BMC Microbiology 14, 36.

Perreten, V., Schwarz, F., Cresta, L., Boeglin, M., Dasen, G., Teuber, M. 1997. Antibiotic resistance spread in food. Nature 389, 801-802.

Ribeiro, T., Abrantes, M., Lopes, M.F.S., Crespo, M.T.B. 2007. Vancomycin-susceptible dairy and clinical enterococcal isolates carry *van*A and *van*B genes. International Journal of Food Microbiology 113, 289-295.

Rivas, B., Marcobal, Á., Muñoz, R. 2005. Improved multiplex-PCR method for the simultaneous detection of food bacteria producing biogenic amines. FEMS - Microbiology Letters 244, 367-372.

Santos, M.H.S. 1996. Biogenic amines: their importance in foods. International Journal of Food Microbiology 29, 213-231.

Schirru, S., Todorov, S.D., Favaro, L., Mangia, N.P., Basaglia, M., Casella, S., Comunian, R., Franco, B.D.G.M., Deiana, P. 2012. Sardinian goat's milk as source of bacteriocinogenic potential protective cultures. Food Control 25, 309-320.

Semedo, T., Santos, M.A., Lopes, M.F.S., Marques, J.J.F., Crespo, M.T.B., Tenreiro, R. 2003. Virulence factors in food, clinical and reference enterococci: a common trait in the genus? Systematic and Applied Microbiology 26, 13-22.

Shankar, V., Baghdayan, A.S., Huycke, M.M., Lindahl, G., Gilmore, M.S. 1999. Infection-derived *Enterococcus faecalis* strains are enriched in *esp*, a gene encoding a novel surface protein. Infection and Immunity 67, 193-200.

Sousa, C.P. 2003. Pathogenicity mechanisms of prokaryotic cells: an evolutionary view. Brazilian Journal of Infectious Diseases 7, 23-31.

Toomey, N., Bolton, D., Fanning, S. 2010. Characterisation and transferability of antibiotic resistance genes from lactic acid bacteria isolated from Irish pork and beef abattoirs. Research in Microbiology 161, 127-135.

Valenzuela, A.S., Omar, N.b., Abriouel, H., López, R.L., Veljovic, K., Cañamero, M.M., Topisirovic, M.K.L., Gálvez, A. 2009. Virulence factors, antibiotic resistance, and bacteriocins in enterococci from artisan foods of animal origin. Food Control 20, 381-385.

Van Tyne, D., Martin, M.J., Gilmore, M.S. 2013. Structure, function, and biology of the *Enterococcus faecalis* cytolysin. Toxins 5, 895-911.

Van Veen, H.W., Konings, W.N. 1998. The ABC family of multidrug transporters in microorganisms. Biochimica et Biophysica Acta (BBA)-Bioenergetics 1365, 31-36.

Vankerckhoven, V., Van Autgaerden, T., Vael, C., Lammens, C., Chapelle, S., Rossi, R., Jabes, D., Goossens, H. 2004. Development of a multiplex PCR for the detection of *asa*1, *gel*E, *cyl*A, *esp*, and *hyl* genes in enterococci and survey for virulence determinants among European hospital isolates of *Enterococcus faecium*. Journal of Clinical Microbiology 42, 4473-4479.

CAPÍTULO 3. Microbial dynamics of a Minas cheese produced with raw goat milk and added of a nisin producer *Lactococcus lactis* subsp. *lactis* GLc05

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As etapas de produção de queijo e monitoramento das populações bacterianas e pH foram realizadas no Departamento de Veterinária e no Departamento de Tecnologia de Alimentos, Universidade Federal de Viçosa, Viçosa, MG, Brasil.

As análises de atividade antimicrobiana, métodos cultura-independentes e pesquisa de aminas biogênicas foram realizadas no Department of Agricultural, Forest and Food Sciences Microbiology and Food Technology area, University of Turin, Grugliasco, Itália.

Title page

Microbial dynamics of a Minas cheese produced with raw goat milk and added of a nisin producer *Lactococcus lactis* subsp. *lactis* GLc05

Running title: Microbial dynamics of a Minas cheese produced with goat milk

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Abstract

Minas cheese is a popular dairy product in Brazil that is traditionally produced using raw or pasteurized cow milk. The present study proposes the use of raw goat milk to produce Minas cheese, added of a nisin producer *Lactococcus lactis* subsp. *lactis* (GLc05) in order to characterize the dynamics of the main microbiota after cheese making and during the ripening and to understand the possible interferences of *L. lactis* subsp. *lactis* GLc05 on autochthonous microbiota; as a safety aspect, the production of biogenic amines (BA) was assessed. Minas cheese was produced in two treatments (A, by adding of GLc05, and B, without adding of *L. lactis* subsp. *lactis* GLc05), in three repetitions (R1, R2, and R3). Culture dependent (direct plating) and independent (rep-PCR and PCR-DGGE) methods were employed to characterize the microbiota of cheeses after cheese making and during ripening. BA amounts were measured using HPLC. A significant decrease in coagulase-positive cocci was observed in the cheeses produced with *L. lactis* subsp. *lactis* GLc05 (A). The rep-PCR and PCR-DGGE highlighted the differences in the microbiota of both cheeses, separating them into two different clusters. *Lactococcus* sp. was found as the main microorganism in both cheeses, and the microbiota of cheese A presented a higher species diversity. Higher concentrations of tyramine were found in both cheeses and, at specific ripening times, the BA amounts in cheese B were significantly higher than in cheese A ($p < 0.05$). In general, it was observed an increase of BA amounts during ripening of both cheeses. The present study demonstrated the interference of the nisin producer *L. lactis* subsp. *lactis* GLc05 in the microbiota dynamics of Minas cheese produced with raw goat milk, and the safety aspects related to BA in this product, highlighting the relevance of monitoring these aspects during production and ripening.

Keywords: Minas cheese, raw goat milk, microbial ecology, PCR-DGGE, rep-PCR, biogenic amines

1. Introduction

Minas cheese is a ripened cheese with milky and buttery notes that is produced by enzymatic coagulation of raw or pasteurized milk, with or without the addition of starter cultures. Minas cheese is the most traditionally cheese produced in the Minas Gerais state, Brazil, being produced by artisanal and industrial plants. The conventional procedures for Minas cheese production are well-known by several Brazilian dairy industries, and in some regions the raw goat milk is employed as the main ingredient. In Brazil, traditional cheeses can be produced with raw milk since they were subjected to at least 60 days of ripening, unless scientific studies demonstrate that fewer days of ripening do not compromise their quality and safety (Brasil, 2013).

Goat milk and its dairy products are widely appreciated because of their nutritional quality, higher digestibility, and therapeutic values in human nutrition (Ribeiro & Ribeiro, 2010). Raw goat milk has a rich autochthonous microbiota that generates specific sensory characteristics in fermented products, such as cheeses. Consumers appreciate these characteristics due to the presence of varied flavor nuances present in these products (Bonetta et al., 2008b; Medina et al., 2011; Montel et al., 2014).

Despite the described sensorial benefits, the main concern in using raw milk is the possible contamination by foodborne pathogens (Brito et al., 2008; Pinto et al., 2009) and the occurrence of spoilage microorganisms that can reduce the shelf life of the product and produce undesirable substances, such as biogenic amines (BA). BA are basic compounds that occur in fermented foods and once ingested at high levels can cause several toxicological problems in the consumers (Bover-Cid & Holzapfel, 1999).

One natural alternative in cheese production is the use of autochthonous lactic acid bacteria (LAB) strains, which are capable of producing antimicrobial substances,

such as bacteriocins. Bacteriocins are antimicrobial peptides, mainly produced by LAB and are active against other bacteria (Cotter et al., 2005). Bacteriocinogenic LAB strains have been extensively studied as biological preservatives in food systems (Biscola et al., 2013; Kruger et al., 2013; Perin et al., 2013; Pingitore et al., 2012; Schirru et al., 2012). However, few studies have investigated the interaction between inoculated LAB strains and autochthonous microbiota in natural matrices, such as cheeses produced with raw milk. LAB may influence the quality and variety of dairy products if they are used as starter cultures for technological purposes or as biological preservatives (Scintu & Piredda, 2007).

Knowledge of the autochthonous microbiota diversity during cheese production and ripening, as well as the influence of LAB inoculation, need to be enhanced. Many studies have already demonstrated the ineffectiveness of using conventional culture-dependent methods to understand the ecology of fermented foods. To overcome its limitations, the use of culture-independent methods have been applied to a variety of dairy products (Arcuri et al., 2013; Delgado et al., 2013; Dolci et al., 2010; Rantsiou et al., 2008) and allowed the simultaneous characterization of whole ecosystems as well as the identification of different species (Cocolin et al., 2013).

The present study aimed to assess the interference of a bacteriocinogenic strain (*Lactococcus lactis* subsp. *lactis* GLc05, nisin producer) in the microbial dynamics after cheese making and during ripening of a Minas cheese produced with raw goat milk. Moreover, production of BA was evaluated as a safety aspect.

2. Material and Methods

2.1. Cheese production

2.1.1. Preparation of *L. lactis* subsp. *lactis* GLc05 culture

L. lactis subsp. *lactis* GLc05 was previously characterized by Perin & Nero (2014), and is able to produce nisin. GLc05 were grown in de Man, Rogosa and Sharpe broth (MRS, Oxoid Ltd., Basingstoke, England) at 35°C for 24 h. The obtained culture was diluted with NaCl 0.85% (w/v) until turbidity equivalent to McFarland scale 1, corresponding to approximately $3x10^8$ colony forming units per mL (CFU/mL). An aliquot of 10 mL of this culture was transferred to 1 L of sterile skimmed milk and incubated at 30°C for 24 h. The obtained culture was used for Minas cheese production, as described in the following.

2.1.2. Minas cheese making

Minas cheese was produced using raw goat milk according to Scholz (1995) and as described in the diagram presented in Figure 1. The cheeses were produced in three independent batches (R1, R2 and R3), and in each batch, the cheeses were produced considering two different treatments (A and B):

- ✓ Cheese A:, by adding the *L. lactis* subsp. *lactis* GLc05 culture to milk before the coagulation step, resulting in a final concentration of 10^6 CFU/mL;
- ✓ Cheese B: control cheese, prepared according Figure 1 without adding the *L. lactis* subsp. *lactis* GLc05.

For both cheeses (A and B), 50 L of raw goat milk were heated at 34°C and added to saturated $CaCl_2$ (20%, w/v) and 2.5 mL of commercial rennet (CHY-MAX® M; CHR Hansen, Hørsholm, Denmark). After 30 min, the curd was cut into cubes with a size of

1 cm^3, and mixed slowly for 40 min. Then, the curd was transferred into circular perforated cheese containers (for 200 g of cheese), pressed for 1 h, and maintained at 10°C overnight. The cheeses were salted in brine with NaCl (20% w/v) at 10°C for 2 h, left to dry for 5 days, packed into plastic bags under vacuum, and ripened at 15°C for 60 days.

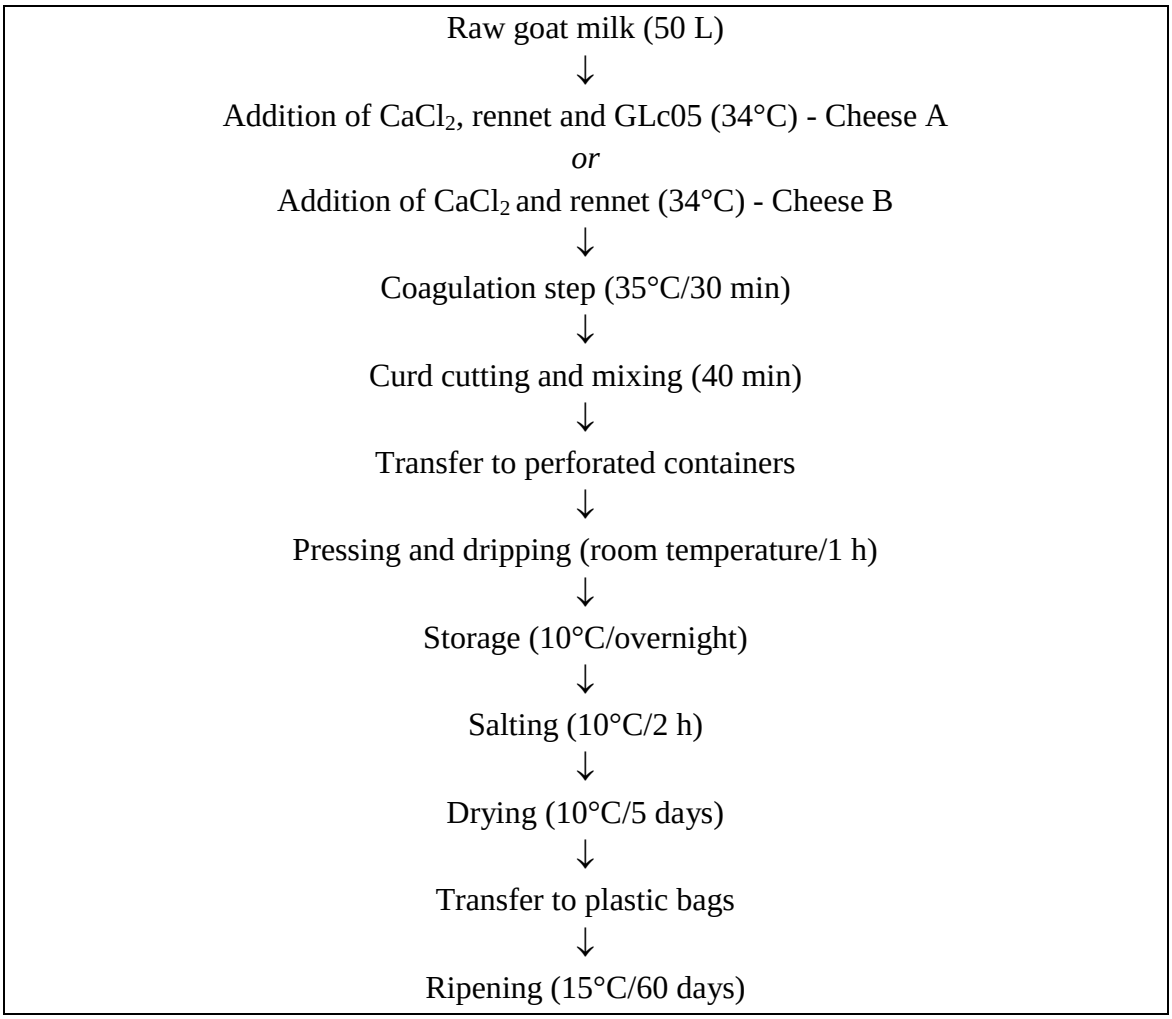

Figure 1. Diagram for Minas cheese production, demonstrating the differences for cheese A and cheese B production in the second step of processing. GLc05: nisin producer *Lactococcus lactis* subsp. *lactis.*

2.2. Microbial dynamics using culture-dependent methods

2.2.1. Microbial analysis and pH values

Samples of cheeses A and B were immediately collected after cheese making

(t = 0), after salting (t = 1 day), and during ripening (every 5 days until 30 days, and after 60 days); the samples were then subjected to microbial analysis. Samples of 25 g of cheese were homogenized in 225 mL of 0.1% saline peptone solution, using a Stomacher (Seward Ltd., Worthing, England) for 1 min, and plated onto selective media for enumeration of the following microbial groups: aerobic mesophilic on Petrifilm™ Aerobic Count (3M, St. Paul, MN, USA) at 35°C for 48 h, coliforms and *Escherichia coli* on Petrifilm™ *Escherichia coli* (3M) at 35°C for 48 h, *Enterococcus* on Kanamycin Aesculin Azide Agar (KAA, Oxoid) at 35°C for 48 h, thermophilic and mesophilic LAB cocci on M17 (Oxoid) at 35 and 42°C for 48 h, thermophilic and mesophilic LAB rods on MRS at 35 and 42°C for 48 h under anaerobiosis, coagulase-negative and coagulase-positive cocci (CNC and CPC, respectively) on Fibrinogen Rabbit Plasma agar (bioMérieux, Marcy l'Etoile, France) at 35°C for 48 h and yeast and molds on Petrifilm™ Yeast and Molds (3M) at 25°C for 5 days.

The pH of each sample was measured in the cheese homogenates in 0.1% saline peptone solution, using a pH meter (HI 221, Hanna Instruments, São Paulo, Brazil).

The results were expressed as log CFU/g and the mean counts were compared by analysis of variance (ANOVA; $p < 0.05$), followed by the Fisher test ($p < 0.05$), to identify significant differences between the results obtained for each cheese during the production and ripening steps, using Statistica 8.0 (StatSoft Inc., Tulsa, OK, USA). At each time of analysis, after collection of samples for microbiological analysis, the remainder of the cheese samples was immediately frozen at -80°C.

2.2.2. Antimicrobial activity

The presence of antimicrobial substances in the cheese matrixes was verified according to Ávila et al. (2006), with modifications. Briefly, frozen samples of cheeses

A and B were thawed, and 5 g were homogenized with 5 mL of 0.02 N HCl in a Stomacher and centrifuged (12,000 × *g*, 20 min, 4°C). The pH of the supernatants was adjusted to pH 6.0 using 1 N NaOH and then submitted to lyophilization. The lyophilized samples were diluted in 200 µL of Ringer solution, and 50 µL of each sample was transferred to a 5 mm well on BHI (Oxoid) semi-solid agar (0.8% w/v agar-agar) inoculated with *Staphylococcus aureus* ATCC 6538 (at a concentration of 10^5 CFU/mL), as the target-microorganism. The samples that presented clear zones around the wells after 24 h at 35°C were considered as positive for the presence of antimicrobial substances.

2.3. Microbial dynamics using culture-independent methods

2.3.1. Nucleic acid extraction

The total DNA was extracted directly from the cheeses samples, in the same times previously described (see section 2.2.1.), according to Rantsiou et al. (2008). Briefly, 10 g of samples were homogenized in 40 mL of Ringer solution, using a Stomacher, for 1 min. Aliquots of 2 mL were centrifuged for 5 min and the pellets were re-suspended in 120 µL of proteinase K buffer [50 mM Tris–HCl, 10 mM EDTA, pH 7.5, 0.5% (w/v) sodium dodecyl sulfate], 25 µL of proteinase K (25 mg/mL, Sigma-Aldrich, St. Louis, MO, USA), and 50 µL of lysozyme (50 mg/mL, Sigma), and incubated at 50°C for 1 h. Samples were transferred to 1.5 mL tubes with glass beads and 150 µL of 2x breaking buffer [4% Triton X-100 (v/v), 2% (w/v) SDS, 200 mM NaCl, 20 mM Tris, pH 8, 2 mM EDTA, pH 8] was dispensed. Phenol-chlorophorm-isoamyl alcohol (300 µL, 25:24:1, pH 6.7; Sigma-Aldrich) was subsequently added before performing three cycles (30 s at 4.5 motion/s) in a bead-beater machine (Fast Prep-24, MP Biomedicals, Solon, OH). Then, 300 µL of TE (10 mM Tris, 1 mM

EDTA) was added to the tubes and centrifuged at 20,000 × *g* for 5 min. The aqueous phase was transferred to a new tube and precipitated with ice-cold absolute ethanol. The nucleic acids were obtained after centrifugation at 20,000 × *g* for 10 min, washed briefly in 70% ethanol, and re-suspended in 50 µL of sterile water. NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA USA) was used to quantify the total DNA extracted, which was diluted to the final concentration of 100 ng/µL.

2.3.2. rep-PCR

The rep-PCR analysis was performed using the total DNA extracted directly from the cheese samples. The PCR reactions were performed according to Gevers et al. (2001), with modifications, using a single primer $(GTG)_5$ (5'-GTG GTG GTG GTG GTG-3'). PCR final concentration contained 1x PCR buffer, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L deoxynucleoside triphosphates, 0.75 U Taq polymerase (Sigma-Aldrich), 50 pMol of the primer, 2 µL DNA (50 ng/µL), and ultrapure PCR water (Promega Corporation, Madison, WI, USA) was added to a final volume of 25 µL. The PCR conditions were: 5 min at 95°C, 30 cycles of 30 s at 95°C; 1 min at 40°C; 8 min at 65°C; and a final extension of 16 min at 65°C. The PCR products were electrophoresed in 2% (w/v) agarose gels for 2 h at a constant voltage of 120 V in 1x Tris/Borate/EDTA buffer (TBE). A 1 Kb DNA ladder (Sigma-Aldrich) was used as a molecular-size marker. Gels were stained using ethidium bromide (0.5 µg/mL, Sigma-Aldrich) and the images were recorded using transilluminator UVIpro Platinum 1.1 Gel Software (Eppendorf, Hamburg, Germany). Fingerprints were analysed using BioNumerics 6.6 (Applied Maths, Sint-Martens-Latem, Belgium). The similarities between the profiles were calculated using the Pearson correlation and the dendrograms were constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

2.3.3. DGGE

PCR of the extracted DNA was performed using the universal primers 338f (5′-ACT CCT ACG GGA GGC AGC AGCAG-3′) and 518r (5′-ATT ACC GCG GCT GCT GG-3′) (Ampe et al., 1999), annealing to the bacterial V3 region of the 16S rRNA gene. A GC clamp (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3′) was attached to the end of 5′ of primer 338f for DGGE analysis. The PCR was performed in a final volume of 25 μL, containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 1.25 U Taq polymerase (Eppendorf), and 0.2 μM of each primer. Two microliters of template DNA were added to the mixture. PCR conditions were: 10 min at 95°C; 35 cycles of 1 min at 95 °C; 1 min at 42°C; 2 min at 72°C; and a final extension of 7 min at 72°C. The PCR products were electrophoresed in 2% (w/v) Tris-acetate-EDTA agarose gels for verification of the PCR reaction.

The Dcode universal mutation detection system (Bio-Rad Laboratories, Hercules, CA, USA) was used for DGGE analysis. Electrophoresis was performed in a polyacrylamide gel (8% w/v acrylamide: bisacrylamide 37.5:1) using a denaturing gradient from 25 to 55% of urea/formamide in a 1x TAE buffer (2 M Tris base, 1 M glacial acetic acid, 50 M EDTA, pH 8). The electrophoresis was performed at a constant voltage of 120 V for 4 h at 60°C, stained in 1x TAE containing 1x SYBR Green I (Sigma-Aldrich), and then analyzed and photographed under UV illumination using UVIpro Platinum 1.1 Gel Software (Eppendorf) (Dolci et al., 2008). Fingerprints were analysed using BioNumerics 6.6 (Applied Maths). The similarities between the profiles were calculated using the Pearson correlation and dendrograms were constructed using UPGMA.

Selected DGGE bands were extracted from the gels, checked by means of DGGE, and sequenced in MWG Biotech in order to identify the species (Dolci et al.,

2008). The obtained sequences were compared to the database of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/genbank), using the Basic Alignment Search Tool (BLAST, p://www.ncbi.nlm.nih.gov/blast.cgi) software.

2.4. Quantification of BA by HPLC

Samples from cheeses A and B were collected after cheese making (t = 0) and after 10, 30, and 60 days of ripening. The BA amounts were quantified after the extraction and derivatization steps reported by Innocente et al. (2007), with modifications.

For extraction, the cheese samples (5 g) were added to 10 mL of 0.1 M HCl and 0.5 mL of a 1 g/L solution of 1,7-diaminoheptane (internal standard, IS) in 0.1 M HCl; they were then homogenized in a Stomacher for 15 min and centrifuged at 1,400 × g for 20 min at 10°C. The supernatant was recovered and the residue was re-extracted using the same procedure. The supernatants were then submitted to the following derivatization process: a 0.5 mL aliquot was added to 150 µL of 0.1 M NaOH, 150 µL of saturated $NaHCO_3$ solution, and 2 mL of 10 mg/mL dansyl chloride solution in acetone and incubated at 40°C for 1 h whilst stirring using a digital pulse mixer (Glas-Col, Terre Haute, USA). At the end of the derivatization reaction, 300 µL of NH_3 were added and the samples were kept at 20°C for 30 min before filtering on PTFE filters (0.45 µm).

BA quantification was performed with a Thermo-Finnigan Spectra System HPLC (Thermo Scientific) equipped with a P2000 binary gradient pump, a SCM 1000 degasser, an AS 3000 automatic injector, and a Finnigan Surveyor PDA Plus detector (PDA, Thermo Scientific). The ChromQuest software 5.0 (Thermo Scientific) was used for instrument control as well as for UV data collection and processing. Separation was

achieved on a C18 RP Lichrosphere 250 × 4.6 mm, 5 μm (Merck Millipore, Darmstadt, Germany) column equipped with a C18 RP Lichrosphere guard column 5 μm (Merck Millipore). The mobile phase was composed of solvent A (ultrapure water) and solvent B (acetonitrile) (Moret & Conte, 1996; Moret et al., 2005). The flow rate was set at 1 mL/min and the injection volume was 20 μL. The elution program was as follows: A 35%, kept isocratic for 6 min; A 25% for 1 min, kept isocratic for 13 min; A 0% for 1 min; A 35% for 1 min, kept in isocratic for 10 min. PDA spectra were recorded in full-scan modality over the wavelength range of 200–600 nm, and quantification was performed by recording the peak area at 254 nm. The calibration curves were constructed by plotting the peak area ratios of each external-to-internal standard versus the external standard concentration. The following external standards were used: 2-phenylethylamine, putrescine, histamine, cadaverine, 1,7-diaminoheptane (IS), tyramine, and spermidine. All standards were of analytical grade and purchased from Sigma Aldrich.

The results were expressed in mg/kg and the mean counts were compared by ANOVA ($p < 0.05$), followed by the Fisher test ($p < 0.05$), to identify significant differences between the results obtained for each cheese during the production and ripening steps, using the software Statistica 8.0 (StatSoft).

3. Results and Discussion

3.1. Microbial dynamics using culture-dependent methods

The mean values of the microbial populations after cheese making and during the ripening of Minas cheese, inoculated (cheese A) or not (cheese B) with nisin producer *L. lactis* subsp. *lactis* GLc05, and the pH values of the samples are reported in Table 1.

Table 1. Mean counts (log CFU/g) and standard deviation of the main microbial populations in Minas cheese inoculated (A) or not (B) with *L. lactis* subsp. *lactis* GLc05 during the production and ripening.

Step	Time (days)	Cheese	pH	Mesophilic aerobes	Coliforms	*Escherichia coli*	Enterococci	Coagulase positive cocci	Coagulase negative cocci	Yeasts and Moulds	LAB cocci 35 °C	LAB cocci 42 °C	LAB bacilli 35 °C	LAB bacilli 42 °C
production	0	A	6.1 ± 0.5	8.0 ± 0.0 Aa	4.2 ± 0.5 Ba	4,1 ± 1.5 Aa	6.0 ± 1.2 Aa	4.3 ± 0.8 Aa	2.3 ± 0.0 Aa	2.7 ± 0.4 Ba	7.6 ± 0.7 Ba	7.0 ± 1.9 Ba	7.6 ± 0.6 Ba	6.89± 0.6 Aa
		B	7.0 ± 0.1	5.4 ± 0.3 Bb	4.4 ± 0.4 Ba	3.0 ± 0.3 Aa	4.4 ± 1.3 Ba	4.7 ± 0.7 Aa	3.6 ± 0.40 Aa	2.8 ± 0.3 Ba	5.3 ± 0.4 Cb	4.9 ± 0.0 Cb	4.8 ± 0.5 Cb	4.6 ± 0.6 Cb
salting	1	A	5.7 ± 0.4	8.3 ± 0.5 Aa	6.3 ± 0.8 Aba	5.7 ± 1.4 Aa	6.6 ± 0.6 Aa	2.9 ± 1.3 Ab	4.5 ± 0.8 Aa	4.0 ± 1.1 ABa	8.2 ± 0.5 ABa	7.9 ± 1.0 Aa	8.0 ± 0.9 ABa	7.2 ± 0.4 Aa
		B	6.2 ± 0.7	7.8 ± 0.2 Ca	6.3 ± 0.5 aAB	5.4 ± 0.7 Aa	5.8 ± 0.4 ABa	5.3 ± 0.2 Aa	4.6 ± 1.3 Aa	4.6 ± 1.0 ABa	7.3 ± 0.7 Ba	7.2 ± 0.7 Ba	7.6 ± 0.4 Ba	6.5 ± 1.4 BCa
ripening	5	A	5.7 ± 0.1	8.7 ± 0.5 Aa	6.9 ± 0.4 Aa	5.7 ± 1.3 Aa	7.0 ± 0.4 Aa	3.3 ± 0.0 Aa	4.4 ± 0.5 Aa	4.0 ± 1.1 ABa	8.5 ± 0.1 ABa	8.6 ± 0.2 Aa	8.4 ± 0.3 ABa	7.5 ± 0.6 Aa
		B	5.5 ± 0.2	8.6 ± 0.4 ACa	7.4 ± 0.4 Aa	6.1 ± 0.9 Aa	6.4 ± 0.2 ABa	5.3 ± 0.3 Aa	5.1 ± 0.7 Aa	4.5 ± 1.6 ABa	8.8 ± 0.5 Aa	8.9 ± 0.3 ABa	9.2 ± 0.2 Aa	7.9 ± 0.5 ABa
	10	A	5.6 ± 0.2	8.8 ± 0.4 Aa	7.1 ± 0.7 Aba	5.8 ± 1.8 Aa	7.1 ± 0.4 Aa	3.2 ± 0.2 Aa	4.8 ± 0.8 Aa	5.4 ± 0.8 Aa	8.5 ± 0.1 ABa	8.5 ± 0.1 Aa	8.5 ± 0.0 ABa	7.6 ± 0.5 Aa
		B	5.4 ± 0.2	8.6 ± 0.4 ACa	7.3 ± 0.7 Aa	6.3 ± 0.8 Aa	6.7 ± 0.5 ABa	5.3 ± 0.4 Aa	5.8 ± 0.2 Aa	5.0 ± 0.3 ABa	9.2 ± 0.2 Aa	8,9 ± 0.7 Aa	9.3 ± 0.1 Aa	8.4 ± 0.8 ABa
	15	A	5.4 ± 0.0	8.8 ± 0.5 Aa	6.7 ± 0.8 Aba	6.3 ± 1.8 Aa	7.4 ± 0.1 Aa	3.0 ± 0.0 Ab	4.9 ± 0.4 Aa	5.7 ± 0.3 Aa	8.8 ± 0.5 Aa	8.6 ± 0.3 Aa	8.7 ± 0.4 Aa	8.2 ± 1.0 Aa
		B	5.4 ± 0.0	9.0 ± 0.5 Aa	7.2 ± 0.7 Aa	6.4 ± 0.8 Aa	7.1 ± 0.5 Aa	5.2 ± 0.0 Aa	5.9 ± 0.5 Aa	5.5 ± 0.4 Aa	9.0 ± 0.5 Aa	9.0 ± 0.3 ABa	9.0 ± 0.5 Aa	8.6 ± 0.4 Aa
	20	A	5.4 ± 0.0	8.7 ± 0.4 Aa	6.2 ± 0.7 Aba	5.8 ± 1.6 Aa	6.5 ± 1.6 Aa	3.8 ± 0.0 Aa	4.5 ± 0.4 Aa	5.4 ± 0.5 Aa	9.0 ± 0.2 Aa	8.6 ± 0.2 Aa	8.9 ± 0.2 Aa	8.4 ± 0.6 Aa
		B	5.4 ± 0.1	9.0 ± 0.2 Aa	7.2 ± 0.6 Aa	6.1 ± 1.1 Aa	7.2 ± 0.5 Aa	5.3 ± 0.5 Aa	5.5 ± 0.6 Aa	5.3 ± 0.5 Aa	9.1 ± 0.3 Aa	8.8 ± 0.3 ABa	9.2 ± 0.3 Aa	8.4 ± 0.7 ABa
	25	A	5.4 ± 0.0	8.9 ± 0.3 Aa	6.4 ± 1.0 Aba	5.3 ± 1.5 Aa	7.7 ± 0.5 Aa	4.2 ± 1.0 Aa	4.8 ± 0.4 Aa	5.1 ± 0.5 Aa	9.0 ± 0.2 Aa	8.5 ± 0.2 Aa	9.1 ± 0.2 Aa	8.6 ± 0.6 Aa
		B	5.4 ± 0.0	8.7 ± 0.2 ACa	7.0 ± 0.8 Aa	5.9 ± 1.3 Aa	7.2 ± 0.3 Aa	5.8 ± 0.5 Aa	5.3 ± 0.8 Aa	5.8 ± 0.4 Aa	8.7 ± 0.1 Aa	8.3 ± 0.1 ABa	8.7 ± 0.2 ABa	8.4 ± 0.3 ABa
	30	A	5.4 ± 0.1	8.9 ± 0.2 Aa	6.2 ± 1.0 Aba	5.5 ± 1.6 Aa	7.6 ± 0.3 Aa	3.5 ± 0.0 Aa	4.4 ± 0.2 Aa	5.4 ± 1.0 Aa	9.1 ± 0.1 Aa	8.6 ± 0.2 Aa	9.0 ± 0.2 Aa	8.4 ± 0.7 Aa
		B	5.4 ± 0.1	8.8 ± 0.4 ACa	7.0 ± 1.0 Aa	5.8 ± 1.0 Aa	7.3 ± 0.7 Aa	4.7 ± 0.3 Aa	5.3 ± 1.0 Aa	5.5 ± 0.8 ABa	8.8 ± 0.3 Aa	8.4 ± 0.4 ABa	8.8 ± 0.4 Aa	8.5 ± 0.7 Aa
	60	A	5.6 ± 0.1	8.2 ± 0.4 Aa	5.6 ± 0.8 Aba	4.6 ± 1.3 Aa	7.0 ± 0.4 Aa	ND	4.5 ± 0.9 Aa	5.3 ± 0.1 ABa	8.3 ± 0.3 ABa	7.7 ± 0.1 Aa	8.4 ± 0.2 ABa	7.9 ± 0.1 Aa
		B	5.6 ± 0.1	8.4 ± 0.3 ACa	5.9 ± 0.5 Aba	5.1 ± 1.1 Aa	7.0 ± 0.4 Aa	3.0 ± 0.0 Aa	5.1 ± 0.5 Aa	5.2 ± 1.1 ABa	8.5 ± 0.2 Aa	7.9 ± 0.4 ABa	8.5 ± 0.1 ABa	8.2 ± 0.4 ABa

Obs.: Capital letters: mean differences of each treatment in different days of production/ripening (Fisher test, $p < 0.05$); lowercase letters: mean difference between treatments, inoculated or not with GLc05 (ANOVA, $p < 0.05$); ND: not detected, counts lower than 10 CFU/g.

The mean counts of mesophilic aerobes, LAB cocci, and bacilli at 35 and 42°C were higher ($p < 0.05$) in cheese A than in cheese B at the time of production ($t = 0$ h, Table 1). One day after production, these counts increased in cheese B making them statistically similar to the counts in cheese A ($p > 0.05$). The mean counts of mesophilic and thermophilic LAB, as well as presumptive lactococci and lactobacilli, did not present relevant differences in Minas cheeses and reaching the highest value between 8 and 9 log CFU/g, after 5 days of ripening (Table 1). Enterococci counts after 60 days of ripening were around 8 log CFU/g in both cheeses A and B (Table 1). Based on these mean values, LAB was the most prevalent microbial group in Minas cheese during ripening.

Coliforms and *E. coli* counts can be considered high in the cheese samples (Table 1). The values were similar to those observed by Moraes et al. (2009) in raw soft cheese, which indicate the importance of ensuring the microbiological quality of the raw milk employed in the production. Even with a decrease of around 1.0 in the pH value, the mean counts of these groups did not decrease. LAB can be considered as the main group responsible for the pH decrease (Table 1), mainly because of the production of lactic acid, as reported by Dolci et al. (2008).

The mean counts of yeasts and molds started to increase one day after production, reaching around 5 log CFU/g after 60 days of ripening. Yeasts could contribute to the final organoleptic characteristics of the cheese with the production of volatile compounds, and also yeasts can metabolize lactic acid to produce NH_3, which raises the pH value and allows salt-tolerant and acid-sensitive bacteria to grow (Montel et al., 2014).

CPC counts in cheese A were significantly lower ($p < 0.05$) than in cheese B, after one day of production ($t = 1$ day) and after 15 days of ripening and was not detected (counts < 10 CFU/mL) after 60 days of ripening (Table 1).

Even though Minas cheese is a typical dairy product in Brazil, there are no standard regulations for its microbiological quality and safety when it is produced with raw milk. The normative instruction nº 30 (Brasil, 2013) does not establishes maximum limits for microbial counts in that cheeses, or even which pathogens must be assessed, which maybe not ensure safety of the product. A major concern related to cheese production is the poor microbiological quality of the raw milk. Inadequate manufacturing practices and refrigeration during production could also allow the contamination and growth of undesirable microorganisms, such as spoilage and pathogens (Ortolani et al., 2010; Perin et al., 2012). Carmo et al. (2002) described a food-poisoning event from *Staphylococcus* strains present in Minas cheese and raw milk in Brazil, demonstrating the importance of controlling the development of these microorganisms in dairy products.

The CPC count in cheese A did not change, which suggested that bacteriocinogenic *L. lactis* subsp. *lactis* GLc05 inoculation may have interfered in the autochthonous microbiota, determining the decrease in the counts of this group. A previous study demonstrated the inhibitory activity of *L. lactis* subsp. *lactis* GLc05 against some *S. aureus* strains in *in vitro* tests (Perin & Nero, 2014), keeping the inhibitory activity including at 15 ºC (Minas cheese ripening temperature; data not shown). In this study, the *in situ* tests with cheeses A and B samples identified the presence of antimicrobial substances capable of inhibiting *S. aureus* growth. The inhibitory activity was detected in cheese A during some ripening steps. However, this inhibition was also recorded in cheese B (data not shown). These findings may be explained by the production characteristics: the cheeses were manufactured using raw milk and other microorganisms may be capable of producing antimicrobial substances against *S. aureus*.

Other studies have also demonstrated the technological potential of

bacteriocinogenic LAB in raw milk, indicating their possible interference in the autochthonous microbiota of this product (Gonzalez et al., 2003; Psoni et al., 2006; Xanthopoulos et al., 2000). This interference can occur in different pathways, such as competition for nutrients and the production of antagonistic substances like lactic acid, diacetyl, hydrogen peroxide, and bacteriocins (Gálvez et al., 2007).

3.2. Microbial dynamics using culture-independent methods

3.2.1. rep-PCR

The dendrograms obtained by rep-PCR clustering of cheeses A and B are presented in Figure 2. rep-PCR is usually employed for clustering bacterial isolates as a previous screening for subsequent identification by sequencing (Cocolin et al., 2011). However, in this study, rep-PCR was considered as a culture-independent method, using the total DNA extracted from the cheeses, to provide an evidence of the differences between the microbiota from cheeses A and cheeses B. Considering 80% as a coefficient of similarity, two main clusters were obtained for each of the three dendrograms generated, one containing the samples from cheese A and the other containing the samples from cheese B (Figure 2). This result indicates that the microbiota from all cheese A samples, independent of production step and ripening time, was different from cheese B samples based on their molecular profiles. Nevertheless, considering that the only difference between cheeses A and B was the inoculation of *L. lactis* subsp. *lactis* GLc05, this result supports the hypothesis that the starter culture added to cheese A was the responsible for the changes in the autochthonous microbiota of Minas cheese.

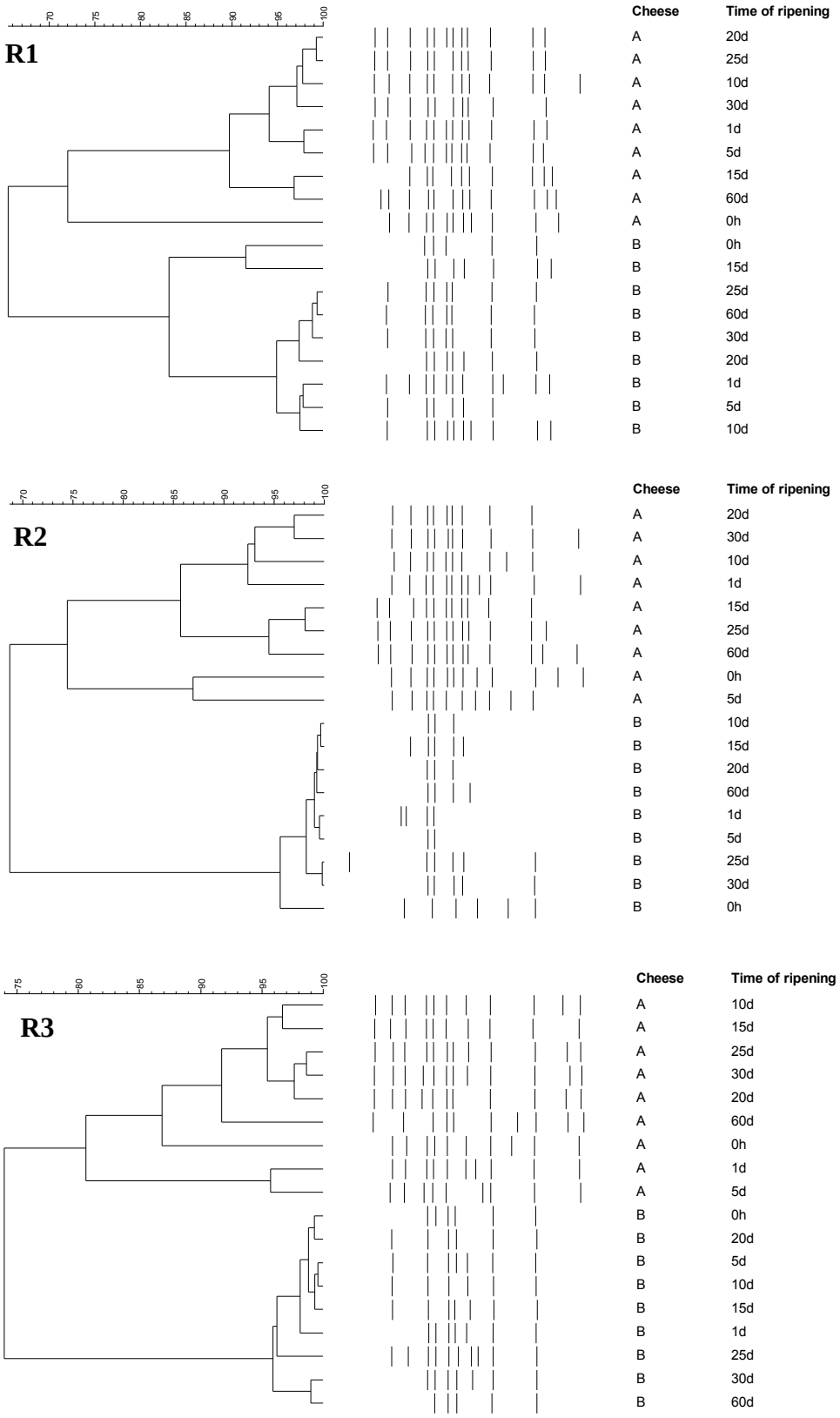

Figure 2. Cluster analysis of rep-PCR fingerprints obtained from Minas cheese produced with raw goat milk inoculated (A) or not (B) with nisin producer *L. lactis* subsp. *lactis* GLc05. The dendrograms were generated for each cheese production (R1, R2 and R3) after cluster analysis of the digitized fingerprints and were derived from UPGMA linkage of Pearson correlation coefficient.

3.2.2. PCR-DGGE

Figure 3 presents the dendrograms obtained by DGGE of cheeses A and B. Considering the results obtained by the three repetitions, the similarity between the cheeses A and B samples was less than 40%, and they were separated into two main clusters (Figure 3). These results confirmed the differences of the microbiota from cheeses A and B, as observed by rep-PCR (Figure 2).

The samples were grouped together depending on the production step and the time of ripening, indicating that the microbiota from cheeses A and B changes during the ripening (Figure 3). The obtained results indicate some differences in the microbiota of the cheeses produced in each repetition: indicating that the Minas cheese microbiota and dynamics could change, depending of the microbial consortia present in the raw milk used for production (data not shown).

The fingerprints from the cheeses A and B are shown in Figure 4. The DNA profile of the *L. lactis* subsp. *lactis* GLc05 strain used for the production of Minas cheese (cheese A) was used as a control. Fifteen bands were selected for sequencing (indicated by numbers in Figure 4). The results of the band identification are reported in Table 2. Cheese samples from the third repetition presented a lower diversity of species and bands (Figure 4), again indicating that the microbiota present in Minas cheese are dependent on the microbiota of the milk (data not shown).

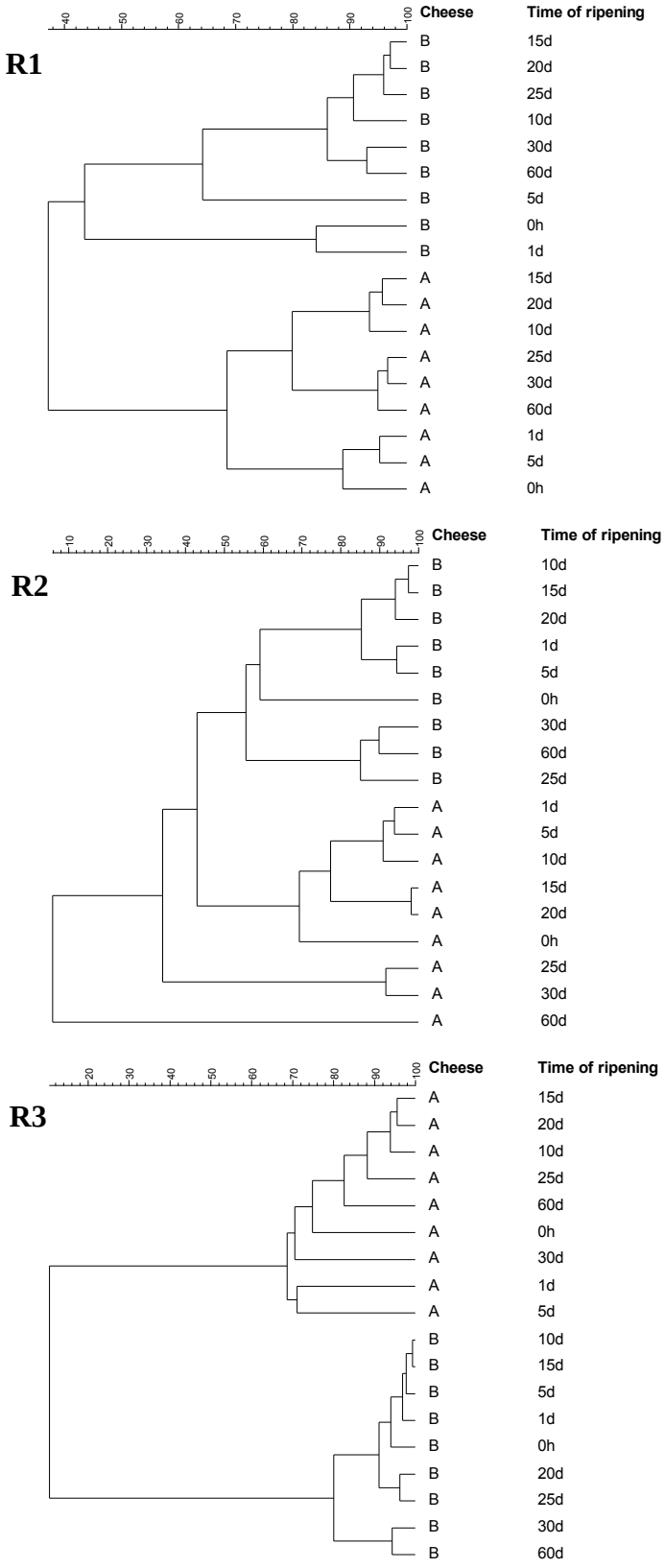

Figure 3. Cluster analysis of PCR-DGGE fingerprints obtained from Minas cheese produced with raw goat milk inoculated (A) or not (B) with nisin producer *L. lactis* subsp. *lactis* GLc05. The dendrograms were generated for each cheese production (R1, R2 and R3) after cluster analysis of the digitized fingerprints and were derived from UPGMA linkage of Pearson correlation coefficient.

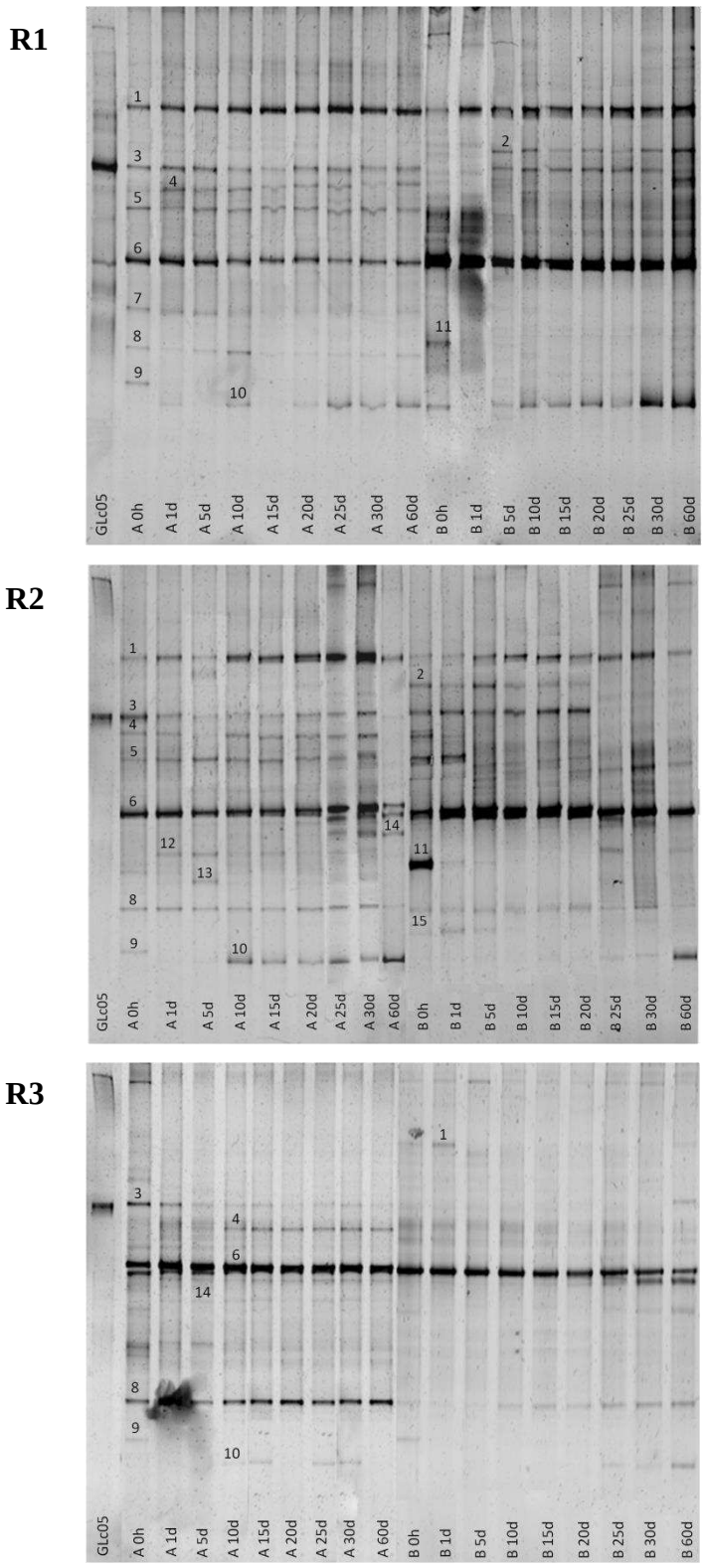

Figure 4. DGGE fingerprintings of the bacterial ecology of the tree production of Minas cheese (R1, R2 and R3) manufactured with raw goat milk and inoculated (lines indicated as "A") or not (lines indicated as "B") with the nisin producer *L. lactis* subsp. *lactis* GLc05 from 0 day of production to 60 days of ripening. The numbers indicate the bands sequenced for molecular identification (results reported in Table 2).

Table 2. Identification of bacterial species present in Minas cheese from the DGGE bands based on the BLAST sequence comparison in GenBank.

Band[a]	Closest sequence relative	% identity[b]	GenBank accession no.
1	*Lactobacillus plantarum*	98%	KF682392.1
2	*Lactococcus lactis*	97%	KF623100.1
3	*Lactococcus lactis*	99%	KF623100.1
4	*Enterococcus faecalis*	99%	AB761302.1
5	*Lactococcus lactis*	99%	KF623100.1
6	*Lactococcus lactis*	99%	KF673548.1
7	*Enterobacter* sp.	99%	AJ564061.1
8	*Lactobacillus* sp.	99%	JX520291.1
9	*Acetobacter* sp.	99%	HF969863.1
10	*Propionibacterium freudenreichii* subsp. *shermanii*	99%	NR_102946.1
11	*Bifidobacterium psychraerophilum*	99%	NR_029065.1
12	*Shigella flexneri*	100%	AM777394.1
13	*Shigella flexneri*	99%	AM777394.1
	Escherichia coli	99%	GU646146.1
14	*Propionibacterium freudenreichii* subsp. *shermanii*	100%	NR_102946.1
15	*Bifidobacterium* sp.	99%	EF990663.1

[a] The numbers correspond to the band numbers in Figure 3.

[b] Percentage of similarity between the sequences obtained from the DGGE band and the sequence of the closest species in the GenBank database.

Four species were exclusively found in cheese A: *Enterococcus faecalis* (band 4, Figure 4), in all repetitions; *Enterobacter* sp. (band 7, Figure 4), in all ripening steps of the first repetition; *Shigella flexneri* (bands 12, 13, Figure 4), only in the second repetition and after one day of production; and *Acetobacter* sp. (band 9, Figure 4), in all repetitions and only after cheese making (t = 0). Instead, only one genera was exclusively found in cheese B: *Bifidobacterium* sp. (bands 11, 15, Figure 4), except in the third repetition, and only after cheese making (t = 0). Only two species, *L. lactis* and *Propionibacterium* sp., were found in both cheeses A and B in all repetitions (Figure 4). In general, cheese A presented a higher number of bands and greater species diversity; this result in cheese A is an interesting finding, because it indicates that *L. lactis* subsp. *lactis* GLc05 enhances the microbial diversity of the cheese, allowing the presence of more species compared to cheese B.

The bands identified as *Shigella flexneri* (bands 12, 13, Figure 4) could be

considered a concern related to the microbiological quality of this product, but it was identified only in R2 and during the first days of ripening (until 5 days, Figure 4). Also it can not ensure that this DNA came from live cells of *S. flexneri*. None of the bands were identified as *S. aureus*, probably because this microorganism was present in cheese samples at concentrations lower than 10^4–10^5 CFU/g (Table 1); bacterial populations that are present below 10^3–10^4 CFU/g cannot be detected by DGGE-PCR (Cocolin et al., 2011). This result demonstrates the relevance of using different culture dependent and independent methods to assess the microbial ecology of food systems, such as the Minas cheese produced with raw goat milk in the present study.

Some studies have characterized the safety and ecology of Minas cheese manufactured with pasteurized milk using only culture-dependent methods (Brito et al., 2008; Moraes et al., 2009; Sant'Ana et al., 2013). The majority of the studies involving Minas cheese are focused on its technological and sensory characteristics or on the occurrence of specific microorganisms (Brito et al., 2008; Nogueira et al., 2005; Pinto et al., 2009; Sant'Ana et al., 2013). To the best of our knowledge, only one study has investigated the ecology of Minas cheese manufactured with raw cow milk using PCR-DGGE (Arcuri et al., 2013). The authors identified that *Streptococcus* sp. and *Lactobacillus* sp. followed by *L. lactis* were the main microorganisms present as autochthonous microbiota. In the present study, *L. lactis* (bands 2, 3, 5, and 6, Figure 4) was the only species present at all ripening times, both in cheeses A and B, and in all repetitions. Bands 2, 5, and 6 (Figure 4) were not present in the *L. lactis* subsp. *lactis* GLc05 profile, indicating the presence of an autochthonous *L. lactis* population coming from the raw goat milk used for cheese production. Band 3 refers to the *L. lactis* subsp. *lactis* GLc05, strain inoculated only in cheese A; however, this band is also present in cheese B (first and second repetitions, Figure 4). This is an expected result, as the *L. lactis* subsp. *lactis* GLc05 strain was isolated from raw goat milk.

3.3. Quantification of BA by HPLC

The BA contents in Minas cheeses A and B are reported in Table 3. Tyramine was present at the highest concentration, with a significant increase ($p < 0.05$) after 30 days of ripening in both cheeses A and B (Table 3). The evidence of a high concentration of tyramine in cheeses, especially in those produced with raw milk, has previously been reported (Bonetta et al., 2008a; Schirone et al., 2011; Spizzirri et al., 2013). Some LAB strains are responsible for tyramine production (Martuscelli et al., 2005; Moraes et al., 2012; Pintado et al., 2008); however, *L. lactis* subsp. *lactis* GLc05 (inoculated in cheese A) is a low tyramine producer (1.19 ± 2.06 mg/kg, data not shown) and cannot be responsible for the high amounts of tyramine in the cheese samples.

Tyramine and histamine have great impact on human health (Bover-Cid & Holzapfel, 1999) and they are described as main BA found in cheese produced with goat milk, while 2-phenylethylamine is usually found at low concentrations (Novella-Rodríguez et al., 2004). In the present study, histamine was detected in cheese A, only after 60 days of ripening, but in low concentration; a non significant increase in the histamine concentration was observed after 30 days of ripening of cheeses B ($p > 0.05$) (Table 3). Histamine has already been recorded at high concentrations in cheeses made with raw milk, demonstrating its relevance towards safety (Bonetta et al., 2008a; Ladero et al., 2008).

Table 3. Mean concentrations (mg/kg) and standard deviation of the biogenic amines in Minas cheese inoculated (A) or not (B) with *L. lactis* subsp. *lactis* GLc05 during the production and ripening.

Biogenic Amine	Cheese	Production and ripening steps (days)			
		0	10	30	60
2-Phenylethylamine	A	45.3 ± 5.2 Ba	135.1 ± 55.8 Aa	94.8 ± 42.2 Aa	135.4 ± 24.9 Ab
	B	203.6 ± 186.7 Ba	135.4 ± 54.6 Ba	244.7 ± 160.7 Ba	592.8 ± 314.0 Aa
Putrescine	A	5.3 ± 8.2 Ab	5.8 ± 5.0 Aa	10.3 ± 8.1 Aa	11.0 ± 10.5 Aa
	B	64.4 ± 48.1 Aa	25.7 ± 24.8 Aa	40.3 ± 46.3 Aa	55.9 ± 63.4 Aa
Histamine	A	ND	ND	ND	25.4 ± 39.4 a
	B	24.7 ± 38.3 ABa	ND	325.8 ± 503.7 Aa	32.9 ± 25.5 ABa
Cadaverine	A	42.9 ± 27.0 Bb	172.8 ± 92.6 Ab	130.3 ± 42.2 Ab	156.2 ± 84.8 Ab
	B	104.9 ± 11.7 Ba	370.9 ± 160.4 ABa	354.8 ± 215.2 ABa	565.4 ± 366.6 Ba
Tyramine	A	1,719.1 ± 110.2 Ba	1,855.5 ± 233.5 Ba	2,523.2 ± 515.9 Aa	2,846.0 ± 547.2 Aa
	B	1,626.9 ± 196.9 Ba	1,780.6 ± 269.3 Ba	2,637.6 ± 162.0 Aa	2,956.9 ± 461.6 Aa
Spermidine	A	45.1 ± 35.2 Ba	68.6 ± 5.1 Aa	75.8 ± 9.5 Aa	81.1 ± 10.5 Aa
	B	56.0 ± 16.9 Ba	70.1 ± 8.6 ABa	75.0 ± 11.2 Aa	77.8 ± 14.1 Aa

Obs. Capital letters: mean differences of each treatment in different days of production/ripening (Fisher test, $p < 0.05$); lowercase letters: mean difference between treatments, inoculated or not with *L. lactis* subsp. *lactis* GLc05 (ANOVA, $p < 0.05$); ND: not detected.

2-phenylethylamine was present at higher levels in cheese B than in cheese A after 60 days of ripening ($p < 0.05$, Table 3). The presence of this BA at high concentrations in cheese was previously described (Martuscelli et al., 2005; Schirone et al., 2011).. Cadaverine was present at lower levels ($p < 0.05$; Table 3) in the cheese A than in cheese B during the production and ripening. Spermidine and putrescine were found in cheeses A and B at low concentrations (Table 3).The presence of *L. lactis* subsp. *lactis* GLc05 in cheese A could play a role in delaying the production of 2-phenylethylamine, cadaverine, and histamine ($p < 0.05$ by comparing cheeses A and B, Table 3).

The presence of BA in cheeses can vary, depending on the type of cheese, precursor amino-acid availability, the ripening time and temperature, the manufacturing process, the quality of raw material, and the microbial ecology (Schirone et al., 2011). The higher BA content in ripened cheeses, compared to fresh ones, is commonly evidenced due to their accumulation over time (Buňková et al., 2013; Loizzo et al., 2013). Higher amounts of BA in cheeses can be originated from the activity of nonstarter LAB or contaminating microbiota, mainly observed when cheeses are manufactured with raw milk. However, Buňková et al. (2013) compared the amount of BA in cheeses produced with both raw and pasteurized milk, and did not find significant differences among them. The safety concentration of BA in foods was not being determinate yet in any regulation, but considering their toxicity to humans, their investigation and control is of utmost importance.

4. Conclusions

L. lactic subsp. *lactis* GLc05 strain, characterized as a nisin producer, was able to control the multiplication of CPC in Minas cheese produced with raw goat milk, and caused a change in the microbial ecology of this cheese during production and ripening.

The obtained results also demonstrated the relevance of monitoring safety aspects during production and ripening of Minas cheese, such as the amounts of BA.

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References

Ampe, F., ben Omar, N., Moizan, C., Wacher, C., Guyot, J.-P. 1999. Polyphasic study of the spatial distribution of microorganisms in Mexican pozol, a fermented maize dough, demonstrates the need for cultivation-independent methods to investigate traditional fermentations. Applied and Environmental Microbiology 65, 5464-5473.

Arcuri, E.F., El Sheikha, A.F., Rychlik, T., Piro-Métayer, I., Montet, D. 2013. Determination of cheese origin by using 16S rDNA fingerprinting of bacteria communities by PCR–DGGE: Preliminary application to traditional Minas cheese. Food Control 30, 1-6.

Ávila, M., Garde, S., Gaya, P., Medina, M., Nuñez, M. 2006. Effect of high-pressure treatment and a bacteriocin-producing lactic culture on the proteolysis, texture, and taste of Hispánico cheese. Journal of Dairy Science 89, 2882-2893.

Biscola, V., Todorov, S.D., Capuano, V.S.C., Abriouel, H., Gálvez, A., Franco, B.D.G.M. 2013. Isolation and characterization of a nisin-like bacteriocin produced by a *Lactococcus lactis* strain isolated from charqui, a Brazilian fermented, salted and dried meat product. Meat Science 93, 607-613.

Bonetta, S., Bonetta, S., Carraro, E., Coisson, J.D., Travaglia, F., Arlorio, M. 2008a. Detection of biogenic amine producer bacteria in a typical Italian goat cheese. Journal of Food Protection 71, 205-209.

Bonetta, S., Bonetta, S., Carraro, E., Rantsiou, K., Cocolin, L. 2008b. Microbiological characterisation of Robiola di Roccaverano cheese using PCR–DGGE. Food Microbiology 25, 786-792.

Bover-Cid, S., Holzapfel, W.H. 1999. Improved screening procedure for biogenic amine production by lactic acid bacteria. International Journal of Food Microbiology 53, 33-41.

Brasil. 2013. Instrução Normativa 30, 2013. About ripening of artisanal cheeses made from raw milk. In: MAPA, (Ed.), 30, vol. 30, 2013, August 8th ed. MAPA, Diário Oficial da União.

Brito, J.R.F., Santos, E.M., Arcuri, E.F., Lange, C.C., Brito, M.A., Souza, G.N., Cerqueira, M.M., Beltran, J.M.S., Call, J.E., Liu, Y. 2008. Retail survey of Brazilian milk and minas frescal cheese and a contaminated dairy plant to establish prevalence, relatedness, and sources of *Listeria monocytogenes* isolates. Applied and Environmental Microbiology 74, 4954-4961.

Buňková, L., Adamcová, G., Hudcová, K., Velichová, H., Pachlová, V., Lorencová, E., Buňka, F. 2013. Monitoring of biogenic amines in cheeses manufactured at small-scale farms and in fermented dairy products in the Czech Republic. Food Chemistry 141, 548-551.

Carmo, L.S., Dias, R.S., Linardi, V.R., Sena, M.J., Santos, D.A., Faria, M.E., Pena, E.C., Jett, M., Heneine, L.G. 2002. Food poisoning due to enterotoxigenic strains of *Staphylococcus* present in Minas cheese and raw milk in Brazil. Food Microbiology 19, 9-14.

Cocolin, L., Alessandria, V., Dolci, P., Gorra, R., Rantsiou, K. 2013. Culture independent methods to assess the diversity and dynamics of microbiota during food fermentation. International Journal of Food Microbiology 167, 29-43.

Cocolin, L., Dolci, P., Rantsiou, K. 2011. Biodiversity and dynamics of meat fermentations: the contribution of molecular methods for a better comprehension of a complex ecosystem. Meat Science 89, 296-302.

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

Delgado, S., Rachid, C.T., Fernández, E., Rychlik, T., Alegría, Á., Peixoto, R.S., Mayo, B. 2013. Diversity of thermophilic bacteria in raw, pasteurized and selectively-cultured milk, as assessed by culturing, PCR-DGGE and pyrosequencing. Food Microbiology 36, 103-111.

Dolci, P., Alessandria, V., Rantsiou, K., Bertolino, M., Cocolin, L. 2010. Microbial diversity, dynamics and activity throughout manufacturing and ripening of Castelmagno PDO cheese. International Journal of Food Microbiology 143, 71-75.

Dolci, P., Alessandria, V., Rantsiou, K., Rolle, L., Zeppa, G., Cocolin, L. 2008. Microbial dynamics of Castelmagno PDO, a traditional Italian cheese, with a focus on lactic acid bacteria ecology. International Journal of Food Microbiology 122, 302-311.

Gálvez, A., Abriouel, H., López, R.L., Omar, N.B. 2007. Bacteriocin-based strategies for food biopreservation. International Journal of Food Microbiology 120, 51-70.

Gevers, D., Huys, G., Swings, J. 2001. Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. FEMS Microbiology Letters 205, 31-36.

Gonzalez, J., Mas, M., Tabla, R., Moriche, J., Roa, I., Rebollo, J.E., Cáceres, P. 2003. Autochthonous starter effect on the microbiological, physicochemical and sensorial characteristics of Ibores goat's milk cheeses. Le Lait 83, 193-202.

Innocente, N., Biasutti, M., Padovese, M., Moret, S. 2007. Determination of biogenic amines in cheese using HPLC technique and direct derivatization of acid extract. Food Chemistry 101, 1285-1289.

Kruger, M.F., Barbosa, M.S., Miranda, A., Landgraf, M., Destro, M.T., Todorov, S.D., Franco, B.D.G.M. 2013. Isolation of bacteriocinogenic strain of *Lactococcus lactis* subsp. *lactis* from rocket salad (*Eruca sativa* Mill.) and evidences of production of a variant of nisin with modification in the leader-peptide. Food Control 33, 467-476.

Ladero, V., Linares, D.M., Fernández, M., Alvarez, M.A. 2008. Real time quantitative PCR detection of histamine-producing lactic acid bacteria in cheese: Relation with histamine content. Food Research International 41, 1015-1019.

Loizzo, M.R., Menichini, F., Picci, N., Puoci, F., Spizzirri, U.G., Restuccia, D. 2013. Technological aspects and analytical determination of biogenic amines in cheese. Trends in Food Science & Technology 30, 38-55.

Martuscelli, M., Gardini, F., Torriani, S., Mastrocola, D., Serio, A., Chaves-López, C., Schirone, M., Suzzi, G. 2005. Production of biogenic amines during the ripening of Pecorino Abruzzese cheese. International Dairy Journal 15, 571-578.

Medina, R., Oliszewski, R., Abeijón Mukdsi, M., Van Nieuwenhove, C., González, S. 2011. Sheep and goat's dairy products from South America: Microbiota and its metabolic activity. Small Ruminant Research 101, 84-91.

Montel, M.-C., Buchin, S., Mallet, A., Delbes-Paus, C., Vuitton, D.A., Desmasures, N., Berthier, F. 2014. Traditional cheeses: Rich and diverse microbiota with associated benefits. International Journal of Food Microbiology 177, 136-154.

Moraes, P.M., Perin, L.M., Todorov, S.D., Silva Jr., A., Franco, B.D.G.M., Nero, L.A. 2012. Bacteriocinogenic and virulence potential of *Enterococcus* isolates obtained from raw milk and cheese. Journal of Applied Microbiology 113, 318-328.

Moraes, P.M., Viçosa, G.N., Yamazi, A.K., Ortolani, M.B.T., Nero, L.A. 2009. Foodborne pathogens and microbiological characteristics of raw milk soft cheese produced and on retail sale in Brazil. Foodborne Pathogens and Disease 6, 245-249.

Moret, S., Conte, L.S. 1996. High-performance liquid chromatographic evaluation of biogenic amines in foods an analysis of different methods of sample preparation in relation to food characteristics. Journal of Chromatography A 729, 363-369.

Moret, S., Smela, D., Populin, T., Conte, L.S. 2005. A survey on free biogenic amine content of fresh and preserved vegetables. Food Chemistry 89, 355-361.

Nogueira, M.C.L., Lubachevsky, G., Rankin, S.A. 2005. A study of the volatile composition of Minas cheese. LWT - Food Science and Technology 38, 555-563.

Novella-Rodríguez, S., Veciana-Nogués, M.T., Roig-Sagués, A.X., Trujillo-Mesa, A.J., Vidal-Carou, M.C. 2004. Evaluation of biogenic amines and microbial counts throughout the ripening of goat cheeses from pasteurized and raw milk. Journal of Dairy Research 71, 245-252.

Ortolani, M.B.T., Moraes, P.M., Perin, L.M., Viçosa, G.N., Carvalho, K.G., Silva Jr., A., Sesma, F., Franco, B.D.G.M., Nero, L.A. 2010. Molecular identification of naturally occurring bacteriocinogenic and bacteriocinogenic-like lactic acid bacteria in raw milk and soft cheese. Journal of Dairy Science 93, 2880-2886.

Perin, L.M., Miranda, R.O., Camargo, A.C., Colombo, M., Carvalho, A.F., Nero, L.A. 2013. Antimicrobial activity of the Nisin Z producer *Lactococcus lactis* subsp. *lactis* Lc08 against *Listeria monocytogenes* in skim milk. Arquivo Brasileiro de Medicina Veterinária e Zootecnia 65, 1554-1560.

Perin, L.M., Moraes, P.M., Viçosa, G.N., Silva Jr., A., Nero, L.A. 2012. Identification of bacteriocinogenic *Lactococcus* isolates from raw milk and cheese capable of producing nisin A and nisin Z. International Dairy Journal 25, 46-51.

Perin, L.M., Nero, L.A. 2014. Antagonistic lactic acid bacteria isolated from goat milk and identification of a novel nisin variant *Lactococcus lactis*. BMC Microbiology 14, 36.

Pingitore, E.V., Todorov, S.D., Sesma, F., Franco, B.D.G.M. 2012. Application of bacteriocinogenic *Enterococcus mundti* CRL35 and *Enterococcus faecium* ST88Ch in the control of *Listeria monocytogenes* in fresh minas cheese. Food Microbiology 32, 38-47.

Pintado, A.I.E., Pinho, O., Ferreira, I.M.P.L.V.O., Pintado, M.M.E., Gomes, A.M.P., Malcata, F.X. 2008. Microbiological, biochemical and biogenic amine profiles of Terrincho cheese manufactured in several dairy farms. International Dairy Journal 18, 631-640.

Pinto, M.S., Carvalho, A.F., Pires, A.C.d.S., Paula, J.C.J., Sobral, D., Magalhães, F.A.R. 2009. Survival of *Listeria innocua* in Minas traditional Serro cheese during ripening. Food Control 20, 1167-1170.

Psoni, L., Tzanetakis, N., Litopoulou-Tzanetaki, E. 2006. Characteristics of Batzos cheese made from raw, pasteurized and/or pasteurized standardized goat milk and a native culture. Food Control 17, 533-539.

Rantsiou, K., Urso, R., Dolci, P., Comi, G., Cocolin, L. 2008. Microflora of Feta cheese from four Greek manufacturers. International Journal of Food Microbiology 126, 36-42.

Ribeiro, A.C., Ribeiro, S.D.A. 2010. Specialty products made from goat milk. Small Ruminant Research 89, 225-233.

Sant'Ana, A.M.S., Bezerril, F.F., Madruga, M.S., Batista, A.S.M., Magnani, M., Souza, E.L., Queiroga, R.C.R.E. 2013. Nutritional and sensory characteristics of Minas fresh cheese made with goat milk, cow milk, or a mixture of both. Journal of Dairy Science 96, 7442-7453.

Schirone, M., Tofalo, R., Mazzone, G., Corsetti, A., Suzzi, G. 2011. Biogenic amine content and microbiological profile of Pecorino di Farindola cheese. Food Microbiology 28, 128-136.

Schirru, S., Todorov, S.D., Favaro, L., Mangia, N.P., Basaglia, M., Casella, S., Comunian, R., Franco, B.D.G.M., Deiana, P. 2012. Sardinian goat's milk as source of bacteriocinogenic potential protective cultures. Food Control 25, 309-320.

Scholz, W. 1995. Elaboración de quesos de oveja y de cabra Acribia SA, Zaragoza.

Scintu, M.F., Piredda, G. 2007. Typicity and biodiversity of goat and sheep milk products. Small Ruminant Research 68, 221-231.

Spizzirri, U.G., Restuccia, D., Curcio, M., Parisi, O.I., Iemma, F., Picci, N. 2013. Determination of biogenic amines in different cheese samples by LC with evaporative light scattering detector. Journal of Food Composition and Analysis 29, 43-51.

Xanthopoulos, V., Polychroniadou, A., Litopoulou-Tzanetaki, E., Tzanetakis, N. 2000. Characteristics of Anevato cheese made from raw or heat-treated goat milk inoculated with a lactic starter. LWT - Food Science and Technology 33, 483-488.

CONCLUSÕES GERAIS

- O leite de cabra cru é importante fonte de novas cepas de BAL bacteriocinogênicas;
- *Lactococcus lactis* isolados de leite de cabra cru foram identificados como capazes de produzir uma nova variante de nisina, com amplo espectro de ação;
- Cepas de BAL bacteriocinogênicas isoladas de leite de cabra cru podem possuir genes relacionados à virulência, resistência a antibióticos e aminas biogênicas e algumas vezes podem ser capazes de expressá-los;
- Cepas de *Lactococcus* spp., consideradas GRAS, podem também apresentar fatores de virulência;
- *L. lactis* subsp. *lactis* GLc05 produtora de nisina foi capaz de controlar a multiplicação da população de cocos coagulase-positivos em queijo Minas produzido com leite de cabra cru;
- *L. lactis* subsp. *lactis* GLc05 foi capaz de causar mudanças na ecologia microbiana de queijo Minas produzido com leite de cabra cru após sua produção e durante maturação;
- Altas concentrações de aminas biogênicas foram encontradas em queijo Minas produzido com leite de cabra cru, revelando importância de garantir a qualidade higiênico-sanitária do leite utilizado;
- Concentrações de aminas biogênicas foram significativamente menores nos queijos Minas adicionados de *L. lactis* subsp. *lactis* GLc05.

APÊNDICES

Tabela 1. Códigos e identificações moleculares de *Enterococcus* bacteriocinogênicos isolados de leite de cabra cru. As identificações foram obtidas a partir do sequenciamento dos genes 16S rRNA e *pheS.*

Codigos antigos	Identificação	Códigos novos
11EN3	*Enterococcus durans*	GEn01
11EN4	*Enterococcus durans*	GEn02
11EN5	*Enterococcus durans*	GEn03
11LB1	*Enterococcus durans*	GEn04
11LC2	*Enterococcus durans*	GEn05
11LC4	*Enterococcus durans*	GEn06
11LC5	*Enterococcus durans*	GEn07
11ST1	*Enterococcus durans*	GEn08
11ST3	*Enterococcus durans*	GEn09
11ST4	*Enterococcus durans*	GEn10
11ST5	*Enterococcus durans*	GEn11
11ST7	*Enterococcus durans*	GEn12
11ST8	*Enterococcus durans*	GEn13
12LB5	*Enterococcus durans*	GEn14
12LC2	*Enterococcus durans*	GEn15
12LC4	*Enterococcus durans*	GEn16
11BA2	*Enterococcus durans*	GEn17
12EN9	*Enterococcus faecalis*	GEn18
13BA21	*Enterococcus faecalis*	GEn19
13LB1	*Enterococcus faecalis*	GEn20
1EN10	*Enterococcus faecalis*	GEn21
1EN23	*Enterococcus faecalis*	GEn22
1EN25	*Enterococcus faecalis*	GEn23
2EN5	*Enterococcus faecalis*	GEn24
2EN7	*Enterococcus faecalis*	GEn25
1ST3	*Enterococcus faecium*	GEn26
11EN8	*Enterococcus faecium*	GEn27
13LC10	*Enterococcus faecium*	GEn28
1EN4	*Enterococcus faecium*	GEn29
2EN24	*Enterococcus faecium*	GEn30
2LC4	*Enterococcus faecium*	GEn31
2LC5	*Enterococcus faecium*	GEn32
13LC6	*Enterococcus hirae*	GEn33

Tabela 2. Códigos e identificações moleculares de *Lactococcus* bacteriocinogênicos isolados de leite de cabra cru. As identificações foram obtidas a partir do sequenciamento dos genes 16S rRNA.

Códigos antigos	Identificação	Códigos novos
13BA7	*Lactococcus lactis*	GLc01
13EN23	*Lactococcus lactis*	GLc02
1LB8	*Lactococcus lactis*	GLc03
13BA1	*Lactococcus lactis* subsp. *lactis*	GLc04
13BA22	*Lactococcus lactis* subsp. *lactis*	GLc05
13BA23	*Lactococcus lactis* subsp. *lactis*	GLc06
13BA25	*Lactococcus lactis* subsp. *lactis*	GLc07
13BA3	*Lactococcus lactis* subsp. *lactis*	GLc08
13BA5	*Lactococcus lactis* subsp. *lactis*	GLc09
13EN2	*Lactococcus lactis* subsp. *lactis*	GLc10
13EN24	*Lactococcus lactis* subsp. *lactis*	GLc11
13EN3	*Lactococcus lactis* subsp. *lactis*	GLc12
13EN4	*Lactococcus lactis* subsp. *lactis*	GLc13
13EN5	*Lactococcus lactis* subsp. *lactis*	GLc14
13EN6	*Lactococcus lactis* subsp. *lactis*	GLc15
13EN7	*Lactococcus lactis* subsp. *lactis*	GLc16
13LC1	*Lactococcus lactis* subsp. *lactis*	GLc17
13LC23	*Lactococcus lactis* subsp. *lactis*	GLc18
13LC5	*Lactococcus lactis* subsp. *lactis*	GLc19
2EN1	*Lactococcus lactis* subsp. *lactis*	GLc20
2EN13	*Lactococcus lactis* subsp. *lactis*	GLc21
2EN4	*Lactococcus lactis* subsp. *lactis*	GLc22
4BA4	*Lactococcus lactis* subsp. *lactis*	GLc23
13LC7	*Lactococcus lactis* subsp. *lactis*	GLc24

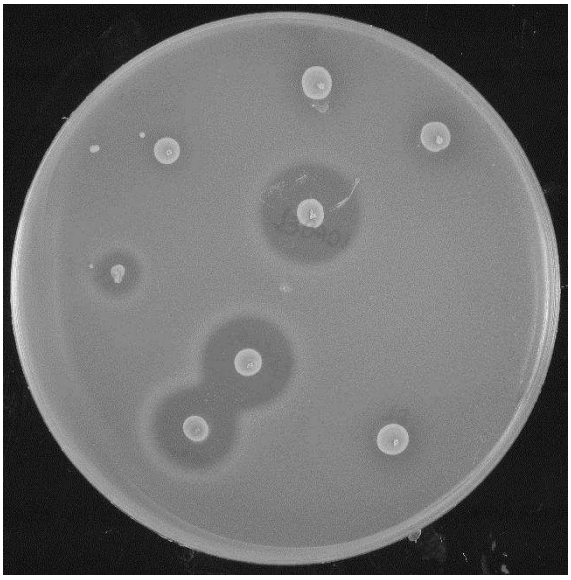

Figura 1. Fotografia de halos de inibição a partir da técnica spot-on-the-lawn. Micro-organismo indicador: *Listeria monocytogenes* ATCC 7644.

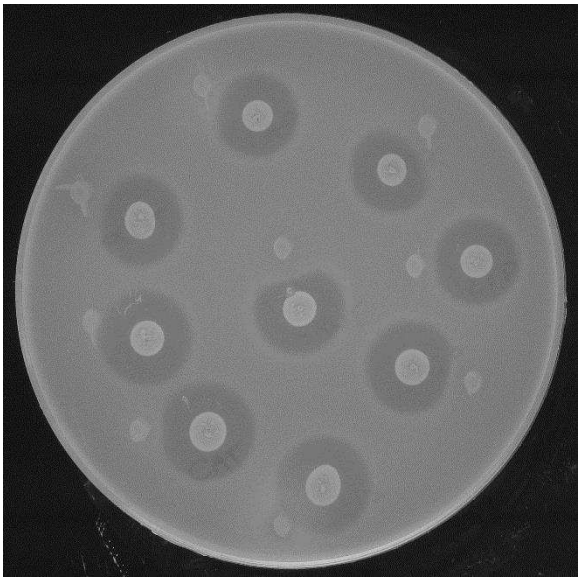

Figura 2. Sensibilidade das substâncias antimicrobianas produzidas pelas cepas de BAL isoladas de leite de cabra cru às enzimas proteinase K a partir da técnica spot-on-the-lawn modificada em MRS. O halo de inibição em formato de meia lua indica a degradação enzimática. Micro-organismo indicador: *Listeria monocytogenes* ATCC 7644.

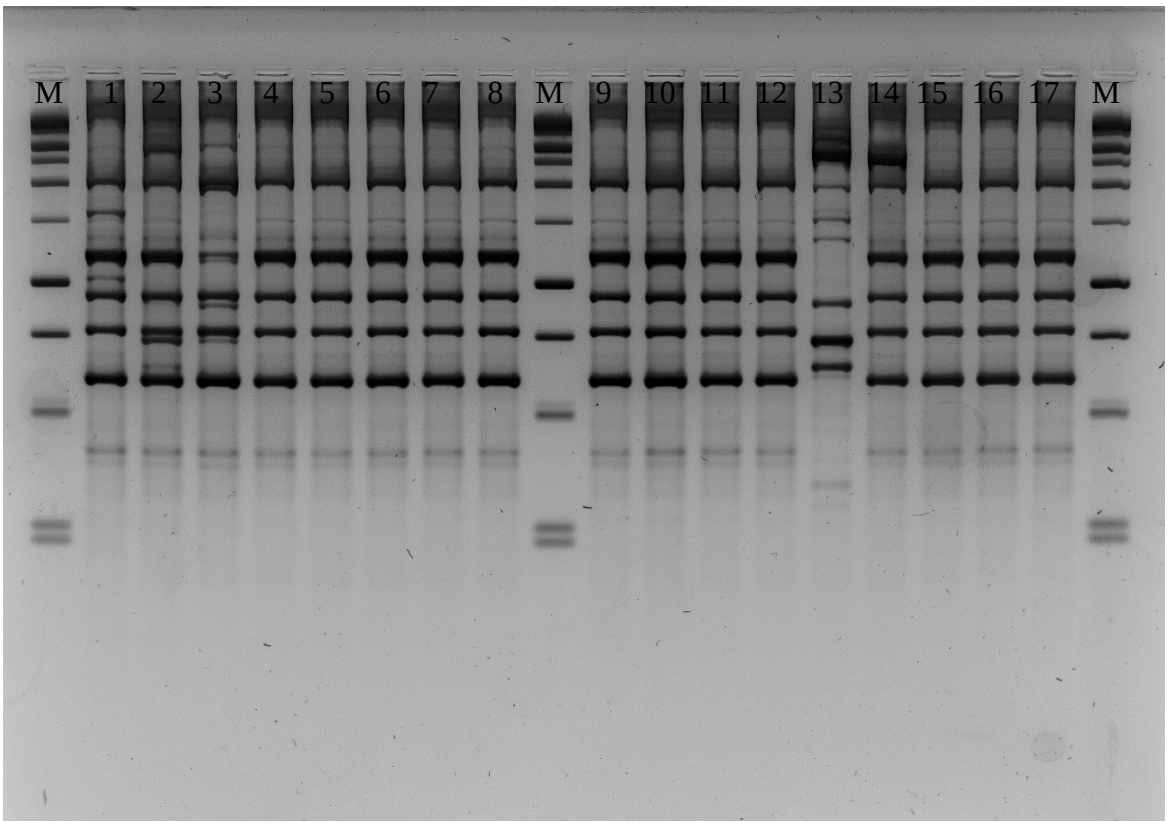

Figura 3. Fotografia de eletroforese em gel de agarose de produtos de rep-PCR. M corresponde ao marcador de peso molecular de 1 kb e os números (1-17) correspondem aos isolados testados.

Tabela 1. Perfil de sensibilidade enzimática das substâncias antimicrobianas produzidas por BAL de leite de cabra cru (+: sensível; -: não sensível).

Códigos	Identifição	Papaína	α-quimotripsina	Proteinase K	α-amilase	Protease	Lipase	Catalase	Tripsina	Lisozima
11EN3	*Enterococcus durans*	+	+	+	-	+	-	-	-	-
11EN4	*Enterococcus durans*	-	+	+	-	+	-	-	-	-
11EN5	*Enterococcus durans*	+	+	+	-	+	+	+	+	-
11LB1	*Enterococcus durans*	+	+	+	-	+	+	-	+	-
11LC2	*Enterococcus durans*	-	+	+	-	+	+	+	+	-
11LC4	*Enterococcus durans*	-	-	+	+	+	+	-	+	-
11LC5	*Enterococcus durans*	+	+	+	-	+	+	-	+	-
11ST1	*Enterococcus durans*	-	+	+	-	-	-	-	+	-
11ST3	*Enterococcus durans*	-	+	+	-	-	-	-	-	-
11ST4	*Enterococcus durans*	-	+	+	-	+	+	-	+	-
11ST5	*Enterococcus durans*	+	+	+	-	+	+	-	+	-
11ST7	*Enterococcus durans*	+	+	-	-	+	+	-	-	-
11ST8	*Enterococcus durans*	+	+	+	-	-	-	-	+	-
12LB5	*Enterococcus durans*	+	-	+	-	+	+	-	+	-
12LC2	*Enterococcus durans*	-	+	+	-	+	+	-	-	-
12LC4	*Enterococcus durans*	+	-	-	-	+	-	-	+	-
11BA2	*Enterococcus durans*	-	-	+	+	+	-	+	+	-
12EN9	*Enterococcus faecalis*	+	+	-	-	-	-	-	-	-
13BA21	*Enterococcus faecalis*	-	-	+	-	+	-	-	+	-
13LB1	*Enterococcus faecalis*	-	+	+	-	+	-	-	+	-
1EN10	*Enterococcus faecalis*	+	-	+	-	-	-	-	+	-
1EN23	*Enterococcus faecalis*	-	+	+	-	+	-	-	+	-
1EN25	*Enterococcus faecalis*	-	+	+	-	+	-	-	+	-
2EN5	*Enterococcus faecalis*	-	+	+	-	+	-	-	+	-
2EN7	*Enterococcus faecalis*	-	-	+	-	+	-	+	-	-
1ST3	*Enterococcus faecium*	-	+	+	-	+	+	-	+	-
11EN8	*Enterococcus faecium*	-	-	-	-	-	-	-	-	-
13LC10	*Enterococcus faecium*	-	+	-	-	+	-	-	-	-

Continuação da Tabela 1

Códigos	Identifição	Papaína	α-quimotripsina	Proteinase K	α-amilase	Protease	Lipase	Catalase	Tripsina	Lisozima
1EN4	*Enterococcus faecium*	-	+	+	-	+	+	-	+	-
2EN24	*Enterococcus faecium*	+	+	+	+	+	+	+	+	+
2LC4	*Enterococcus faecium*	+	+	+	+	+	+	+	+	+
2LC5	*Enterococcus faecium*	+	+	+	-	+	-	-	-	-
13LC6	*Enterococcus hirae*	-	-	+	-	+	+	+	+	-
13BA7	*Lactococcus lactis*	-	+	-	-	+	+	-	+	-
13EN23	*Lactococcus lactis*	-	-	+	-	+	-	-	-	-
1LB8	*Lactococcus lactis*	+	-	-	-	+	-	-	-	-
13BA1	*Lactococcus lactis* subsp. *lactis*	-	-	+	+	+	+	-	+	-
13BA22	*Lactococcus lactis* subsp. *lactis*	+	+	+	-	+	+	-	+	-
13BA23	*Lactococcus lactis* subsp. *lactis*	+	+	+	-	+	-	-	+	-
13BA25	*Lactococcus lactis* subsp. *lactis*	+	+	+	-	+	+	-	+	-
13BA3	*Lactococcus lactis* subsp. *lactis*	+	+	+	-	+	+	-	+	-
13BA5	*Lactococcus lactis* subsp. *lactis*	+	-	+	-	+	+	-	+	-
13EN2	*Lactococcus lactis* subsp. *lactis*	-	+	+	-	+	-	+	+	-
13EN24	*Lactococcus lactis* subsp. *lactis*	+	+	+	-	+	+	+	+	-
13EN3	*Lactococcus lactis* subsp. *lactis*	+	-	+	-	+	+	-	+	-
13EN4	*Lactococcus lactis* subsp. *lactis*	-	+	+	-	+	-	-	-	-
13EN5	*Lactococcus lactis* subsp. *lactis*	-	-	+	-	+	-	-	-	-
13EN6	*Lactococcus lactis* subsp. *lactis*	-	+	+	-	+	-	-	-	-
13EN7	*Lactococcus lactis* subsp. *lactis*	+	-	+	-	+	+	-	-	-
13LC1	*Lactococcus lactis* subsp. *lactis*	-	+	+	-	+	+	+	-	-
13LC23	*Lactococcus lactis* subsp. *lactis*	-	-	+	-	+	-	-	+	-
13LC5	*Lactococcus lactis* subsp. *lactis*	-	+	+	-	+	+	+	+	-
2EN1	*Lactococcus lactis* subsp. *lactis*	-	-	-	-	-	-	-	-	-
2EN13	*Lactococcus lactis* subsp. *lactis*	-	-	-	-	-	-	-	-	-
2EN4	*Lactococcus lactis* subsp. *lactis*	-	-	-	-	+	-	-	-	-
4BA4	*Lactococcus lactis* subsp. *lactis*	+	+	+	+	+	+	-	+	-
13LC7	*Lactococcus lactis* subsp. *lactis*	-	+	+	+	+	+	-	+	-

Capítulo 2

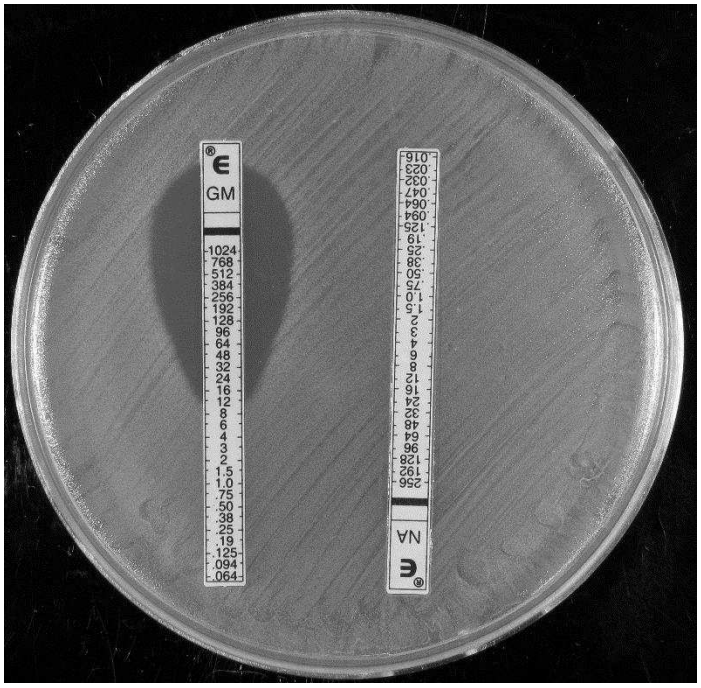

Figura 1. Resistência a antibióticos apresentada pelas cepas de BAL isoladas de leite de cabra cru usando Etest® (BioMérieux SA, Marcy l'Etoile, France). Considerando a formação de halo ao redor das tiras, as concentrações inibitórias mínimas (MIC) foram estimadas (µg/mL) para cada um dos antibióticos contra cada cepa. GM: gentamicina; NA: ácido nalidíxico.

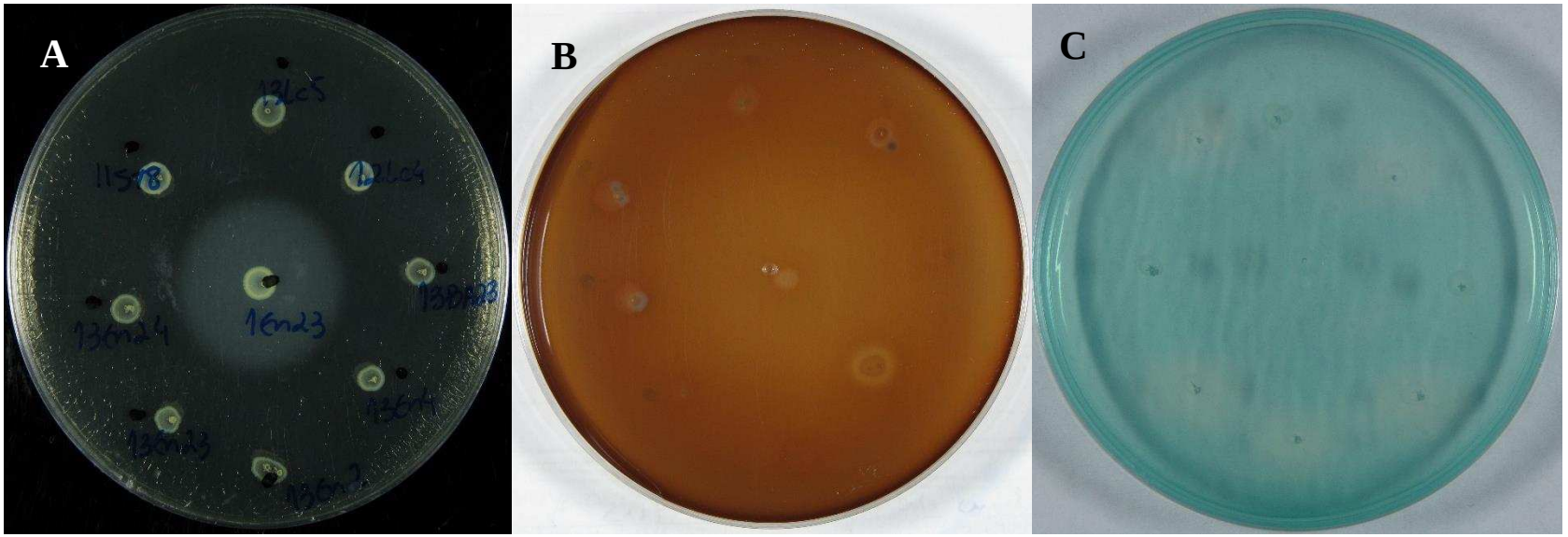

Figura 2. Testes fenotípicos dos fatores de virulência apresentados por cepas de BAL isoladas de leite de cabra cru. A: produção de gelatinase, a formação de halos opacos ao redor da cultura indica a hidrólise da gelatina; B: atividade hemolítica, a formação de halos ao redor da cultura indica hemólise total ou parcial; C: produção de DNAse, indicada pela formação de halos claros ao redor da cultura.

Capítulo 3

Tabela 1. Contagens das populações microbianas presents em queijo Minas inoculado (A) ou não (B) da cepa *L. lactis* subsp. *lactis* GLc05 após a produção e durante maturação.

Trat.	Rep.	Etapas	Aerobios mesófilos	Coliformes	*E. coli*	Enterococci	CPC	CNC	fungos/ leveduras	cocci 35°C	cocci 42°C	bacilli 35°C	bacilli 42°C
A	1	cheese	58000000	472000	552000	1800000	0	100000	46700	50000000	5000000	10000000	9000000
B	1	cheese	72000000	796000	156000	1600000	210000	620000	43100	4000000	3800000	30000000	120000
A	2	cheese	450000000	17400000	9900000	2100000	100	4000	600	370000000	285000000	273000000	46000000
B	2	cheese	35000000	7400000	1500000	330000	133000	2000	4200	17280000	9980000	25280000	5220000
A	3	cheese	309000000	820000	20000	22000000	7000	101000	41000	283000000	310000000	390000000	12000000
B	3	cheese	84900000	1560000	90000	482000	261000	44000	412000	118200000	88000000	109800000	57200000
A	1	cheese, 5d	1350000000	3700000	100000	5300000	0	90000	500	494500000	463000000	536500000	123500000
B	1	cheese, 5d	540000000	13700000	800000	2060000	310000	640000	600	968000000	844000000	876000000	19750000
A	2	cheese, 5d	380000000	21400000	16400000	5700000	2000	10000	33000	269000000	420000000	117000000	38000000
B	2	cheese, 5d	154800000	72500000	10500000	4420000	94000	40000	126000	1460000000	480000000	1624000000	138000000
A	3	cheese, 5d	297000000	6400000	100000	23850000	0	16000	51000	311500000	229500000	325500000	7000000
B	3	cheese, 5d	740000000	11900000	200000	2280000	330000	60000	536000	187500000	1588000000	2200000000	142000000
A	1	cheese, 10d	1670000000	5700000	300000	4500000	2000	78000	34000	368000000	338000000	326000000	75000000
B	1	cheese, 10d	290000000	13500000	900000	3200000	120000	540000	50000	1600000000	1380000000	1600000000	68000000
A	2	cheese, 10d	550000000	75000000	55000000	18300000	1000	8000	995000	290000000	320000000	279000000	58000000
B	2	cheese, 10d	947000000	121000000	17000000	19800000	500000	440000	86000	2990000000	2930000000	2910000000	1630000000
A	3	cheese, 10d	318500000	4020000	20000	21400000	2000	318000	359000	239500000	246500000	310000000	11000000
B	3	cheese, 10d	166000000	5200000	500000	2645000	100000	1140000	189000	1530000000	135000000	1750000000	104500000
A	1	cheese, 15d	1560000000	1660000	0	32800000	0	138000	936000	1730000000	640000000	1360000000	1020000000

Continuação da Tabela 1

Trat.	Rep.	Etapas	Aerobios mesófilos	Coliformes	*E. coli*	Enterococci	CPC	CNC	fungos/ leveduras	cocci 35°C	cocci 42°C	bacilli 35°C	bacilli 42°C
B	1	cheese, 15d	1390000000	9000000	2000000	22400000	150000	1510000	964000	1600000000	780000000	860000000	490000000
A	2	cheese, 15d	770000000	37600000	36400000	33600000	0	25000	609000	700000000	480000000	580000000	207000000
B	2	cheese, 15d	2800000000	84000000	16000000	29300000	0	1240000	203000	2820000000	2270000000	2930000000	950000000
A	3	cheese, 15d	198500000	2000000	100000	18800000	1000	115000	270000	207500000	201000000	210000000	16000000
B	3	cheese, 15d	261000000	4900000	400000	3000000	130000	230000	200000	297000000	540000000	304500000	162500000
A	1	cheese, 20d	1330000000	5100000	500000	51300000	6000	46000	119000	1520000000	540000000	1510000000	1050000000
B	1	cheese, 20d	1050000000	31000000	1000000	34200000	530000	1280000	568000	1210000000	530000000	1380000000	680000000
A	2	cheese, 20d	318500000	0	34000000	13950000	0	11900	970000	1000000000	520000000	800000000	326500000
B	2	cheese, 20d	1660000000	44000000	19000000	27700000	330000	80000	71000	2360000000	1390000000	2660000000	620000000
A	3	cheese, 20d	257000000	470000	20000	60000	0	64000	110000	520000000	200000000	500000000	60000000
B	3	cheese, 20d	570000000	3800000	100000	4400000	50000	350000	170000	690000000	350000000	770000000	47000000
A	1	cheese, 25d	1780000000	1010000	70000	153000000	3000	41000	36000	1570000000	445000000	1960000000	1180000000
B	1	cheese, 25d	790000000	38100000	100000	29000000	1350000	1450000	1630000	630000000	187000000	770000000	428500000
A	2	cheese, 25d	336500000	19800000	19600000	24000000	0	43000	590000	374500000	240000000	381500000	123500000
B	2	cheese, 25d	870000000	34000000	8000000	58000000	70000	160000	170000	1040000000	169500000	880000000	570000000
A	3	cheese, 25d	378000000	620000	10000	18850000	0	38000	307000	750000000	410000000	980000000	69000000
B	3	cheese, 25d	600000000	1310000	200000	7300000	231000	117000	250000	510000000	198000000	550000000	216000000
A	1	cheese, 30d	1150000000	540000	80000	71000000	3000	28000	50000	1760000000	308500000	1440000000	1170000000
B	1	cheese, 30d	1060000000	46400000	400000	79000000	40000	2610000	1230000	1210000000	243000000	1170000000	1200000000
A	2	cheese, 30d	960000000	22800000	22000000	37000000	0	15000	1120000	950000000	610000000	770000000	320000000
B	2	cheese, 30d	1030000000	25000000	8000000	35000000	108000	37000	80000	920000000	520000000	1040000000	490000000
A	3	cheese, 30d	440000000	310000	20000	22400000	0	30000	0	1050000000	307500000	662000000	52000000
B	3	cheese, 30d	252000000	660000	90000	3800000	37000	92000	0	297500000	83000000	230000000	50000000
A	1	cheese, 60d	335000000	709000	9000	20000000	0	162000	216000	370000000	35900000	370000000	71200000

Continuação da Tabela 1

Trat.	Rep.	Etapas	Aerobios mesófilos	Coliformes	*E. coli*	Enterococci	CPC	CNC	fungos/ leveduras	cocci 35°C	cocci 42°C	bacilli 35°C	bacilli 42°C
B	1	cheese, 60d	320000000	712000	12000	11000000	0	358000	900000	340000000	100800000	370000000	200000000
A	2	cheese, 60d	176500000	1280000	1270000	14000000	0	3000	168000	130000000	65000000	174000000	102000000
B	2	cheese, 60d	320500000	2080000	1670000	24000000	0	34000	24000	560000000	182000000	410000000	297000000
A	3	cheese, 60d	59000000	50000	5000	3800000	0	69000		116000000	60000000	320500000	73500000
B	3	cheese, 60d	108000000	260000	70000	3600000	1000	114000		210500000	37000000	225500000	48000000

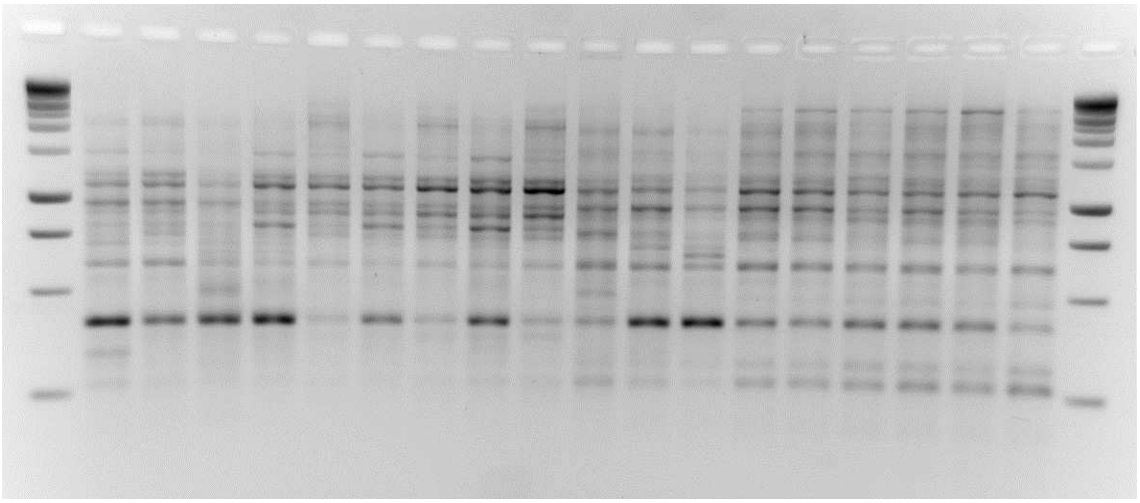

Figura 1. Fotografia de eletroforese em gel de agarose de produtos de rep-PCR de amostras de queijo Minas produzidos experimentalmente.