ANDRESSA FUSIEGER

TECHNOLOGICAL FEATURES AND ABILITY TO PRODUCE NISIN BY *Lactococcus lactis* subsp. *lactis* bv. diacetylactis OBTAINED FROM DAIRY ENVIRONMENT

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, para obtenção do título de *Magister Scientiae.*

VIÇOSA
MINAS GERAIS – BRASIL
2019

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T
F993t
2019

Fusieger, Andressa, 1995-
Technological features and ability to produce nisin by *Lactococcus lactis* subsp. *lactis* bv. diacetylactis obtained from dairy environment / Andressa Fusieger. – Viçosa, MG, 2019.
xiii, 100 f. : il. (algumas color.) ; 29 cm.

Texto em inglês e português.
Orientador: Luís Augusto Nero.
Dissertação (mestrado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Bactérias do ácido lático. 2. Fermentação. 3. Diacetil. 4. Bacteriocinas. I. Universidade Federal de Viçosa. Departamento de Tecnologia de Alimentos. Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos. II. Título.

CDD 22. ed. 664.001579

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APROVADA: 19 de fevereiro de 2019.

Luciano dos Santos Bersot

Antônio Fernandes de Carvalho
(Coorientador)

Luís Augusto Nero
(Orientador)

Aos meus pais Nair e Paulo.

Aos meus avós Maria Nelsi e Isidoro.

DEDICO

AGRADECIMENTOS

Agradeço a Deus e a Santo Antônio, pela luz nos dias de escuridão, pela esperança nos momentos de dúvida e pela paciência nas horas turbulentas. Com o Vosso amor sustentei a luta de cada dia.

Aos meus pais, Nair e Paulo, pela formação do meu caráter e apoio incondicional. Por compreenderem minhas idas e voltas, acreditarem e estarem sempre ao meu lado para a concretização dos meus ideais, apesar da distância e saudade. Obrigada por nunca terem desistido de mim, amo vocês!

Aos meus familiares, pelo incentivo, risadas e renovação de energia nos finais de ano na casa dos avós Fank. À tia Vivia pelo carinho e por nunca medir esforços para me ajudar e estar ao meu lado. À Chica, nosso xodó, por me aguardar ansiosamente a cada retorno e companhia fiel. Agradeço a minha guerreira *vói* Maria Nelsi, pelo seu exemplo de perseverança, ensinamentos, orações e pelo orgulho que transborda de seus olhos a cada conquista minha.

Ao Prof. Dr. Luís Augusto Nero, pela disponibilidade de orientação, confiança, oportunidades e pelo exemplo de profissionalismo. Obrigada pelas contribuições com ideias e sugestões e pelos ensinamentos imprescindíveis para a realização deste trabalho.

Ao estimado Prof. Dr. Antônio Fernandes de Carvalho, pela orientação, confiança, suporte, paciência e bom humor. Pelos desafios impostos a fim de me incentivar a correr atrás dos objetivos. Obrigada pela cumplicidade e pelo exemplo que és, como pesquisador e como pessoa.

À Luana Martins Perin, pelas contribuições e sugestões na execução dos experimentos, principalmente nos testes moleculares e sequenciamento.

À Universidade Federal de Viçosa e ao Departamento de Tecnologia de Alimentos, pela formação e pela oportunidade de realização deste trabalho. À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pelo apoio financeiro.

Ao Centro Internacional de Recursos Microbianos de Bactérias de Interesse Alimentar (CIRM/BIA) do Instituto de Pesquisa Agronômica (INRA/França) pela concessão das culturas de referência.

À Mayra Carla de Freitas Martins, pelos ensinamentos e oportunidades. Obrigada pela amizade, inspiração e conselhos.

Aos colegas e amigos da equipe do InovaLeite/UFV, InsPOA/UFV e agregados, pelo companheirismo no dia-a-dia laboratorial e colaboração. Pela troca de conhecimentos dentro e fora do laboratório, momentos de alegria e descontração.

À minha primeira professora de Microbiologia de Alimentos, Prof.ª Dr. Ana Eucares von Laer, por onde tudo começou.

À Milimani, uma irmã que a vida colocou no meu caminho. Pela companhia imprescindível, festas, churrascos, mates e amizade sincera. Pela pessoa verdadeira que és e pelo carinho, paciência, conselhos e compreensão em todos os momentos. Por estar ao meu lado vivendo, sonhando, aprendendo e conquistando.

À Camila, companheira leal de laboratório, pela amizade, prestatividade e dedos cruzados durante a execução das análises.

À Palloma, pelo convívio no 1001, amizade, plenitude, incentivo, cafés e tapiocas. Foi um prazer morar com você.

Aos meus grandes amigos, Leila e Mathias, por fazerem parte da minha vida, pelo apoio, afeto e por sempre estarem conectados e presentes.

Por fim, agradeço a todos que contribuíram de uma forma ou outra, seja para a concretização deste trabalho ou em prol do meu crescimento intelectual e pessoal. Aos que estiveram ao meu lado, nos momentos bons, mas principalmente nos momentos difíceis, de dúvida e de questionamento, meu mais sincero obrigada.

Vielen Dank!!!

Frida.

"Só quem parte é quem sabe da dor de deixar o seu pago e sua gente,
as lembranças rebrotam ao redor, só o forte consegue ir em frente.
Nos pessuêlos vão laços de afeto e a honra de ser o que são,
os centauros da banda do Sul, povo guapo criado em galpão.
Ao chegar no torrão de seu gosto vão semeando alegria e respeito,
o trabalho em seguida da fruto e o fruto é um consolo pro peito."

Os Monarcas

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RESUMO

FUSIEGER, Andressa, M.Sc., Universidade Federal de Viçosa, fevereiro de 2019. **Características tecnológicas e habilidade de produzir nisina por cepas de *Lactococcus lactis* subsp. *lactis* bv. diacetylactis obtidas do ambiente lácteo.** Orientador: Luís Augusto Nero. Coorientador: Antônio Fernandes de Carvalho.

Cepas de *Lactococcus lactis* subsp. *lactis* bv. diacetylactis são utilizadas na indústria de laticínios para produzir acetoína e diacetil, que conferem características sensoriais específicas a derivados lácteos fermenados. A produção de bacteriocinas pode ser uma característica benéfica adicional apresentada por algumas cepas. A identificação e caracterização de novas cepas de *Lactococcus* podem revelar atributos distintos que apresentam grande potencial tecnológico para a indústria de laticínios. Este estudo teve como objetivo caracterizar o potencial tecnológico e bacteriocinogênico de cepas de *L. lactis* subsp. *lactis* bv. diacetylactis obtido a partir de sistemas de produção de leite. Vinte e três cepas de *L. lactis* subsp. *lactis* isoladas de leite de vaca, cabra e búfala, creme de leite de vaca, queijos artesanais da região amazônica e da Ilha de Marajó, silagem de amendoim forrageiro e silagem de capim foram utilizados neste estudo. As cepas foram identificadas em nível biovar (produção de acetoina e diacetil, além de PCRs específicas) e similariedade genética por rep-PCR e quanto ao seu potencial tecnológico: padrões de lactofermentação, atividade proteolítica extracelular, capacidade de acidificação e resistência ao NaCl. Posteriormente, as cepas foram submetidas à PCR para detectar genes relacionados à bacteriocinas (nisina, lacticina 481 e 3147, lactococina A e 972), e o operon relacionado a produção de nisina foi sequenciado para identificação de variações na produção dessa bacteriocina. Os sobrenadantes livres de células (CFS) de cepas positivas para os genes relacionados a produção de nisina foram testados pelo ensaio *spot-on-the-lawn* frente a um painel de 16 alvos (*Listeria monocytogenes* - 4, *L. innocua* - 1, *Staphylococcus aureus* - 6, *Lactobacillus sakei* - 1, *L. lactis* - 4). Curvas de crescimento de 4 alvos microbianos (*L. monocytogenes* - 2, *S. aureus* - 2), isoladamente e na presença do CFS dos produtores de nisina, foram obtidas por densidade óptica (λ = 650 nm). A partir da coleção de culturas de 23 cepas de *L. lactis* subsp. *lactis*, 15 cepas apresentaram resultados moleculares e fenotípicos que permitiram sua identificação como *L. lactis* subsp. *lactis* bv. diacetylactis. A análise de rep-PCR demostrou 11 perfis genéticos diferentes com similariedade inferior a 90%, indicando um alto nível de diversidade

entre as 15 cepas identificadas como *L. lactis* subsp. *lactis* bv. diacetilactis. A caracterização tecnológica indicou que duas cepas não apresentaram habilidades de coagulação e 13 foram positivas para a atividade proteolítica extracelular. Muitas cepas se mostraram eficientes para acidificar leite, e duas cepas apresentaram alta capacidade de acidificação, resultando em uma redução do pH em 2 unidades após 24h. O ensaio de tolerância ao NaCl revelou que todas as cepas foram capazes de se multiplicar em diferentes concentrações (0, 2, 4, 6, 8 e 10%); entretanto, cepas isoladas de nichos não lácteos mostraram uma tolerância ainda maior ao NaCl (10%). Para os genes relacionados à bacteriocina, oito cepas apresentaram resultados positivos apenas para *nisA*, e apenas uma cepa (SBR4) apresentou o operon completo da nisina, confirmado pelo sequenciamento como similar à nisina Z. Apenas a cepa SBR4 apresentou atividade inibitória pelo ensaio *spot-on-the lawn* frente os 16 alvos microbianos. Curvas de crescimento de alvos selecionados confirmaram a atividade inibitória da cepa SBR4, especialmente contra *S. aureus*, indicando seu potencial para produção de nisina. Deste modo, esses resultados indicam que as cepas estudadas apresentam características tecnológicas importantes para a indústria de alimentos e que sua aplicação é viável para a produção de alimentos fermentados. Considerando as características tecnológicas de *L. lactis* subsp. *lactis* bv. diacetylactis SBR4, além de sua capacidade de produzir nisina, essa pode ser usada para constituir uma cultura starter ou mista, sendo uma linhagem a ser considerada na indústria de laticínios como uma ferramenta bioconservadora e biotecnológica.

ABSTRACT

FUSIEGER, Andressa, M.Sc., Universidade Federal de Viçosa, February, 2019. **Technological features and ability to produce nisin by *Lactococcus lactis* subsp. *lactis* bv. diacetylactis obtained from dairy environment.** Advisor: Luís Augusto Nero. Co-advisor: Antônio Fernandes de Carvalho.

Lactococcus lactis subsp. *lactis* bv. diacetylactis strains are used in the dairy industry to produce acetoin and diacetyl, substances that are responsible for conferring organoleptical characteristics to some fermented foods. Bacteriocin production can be an additional beneficial feature presented by some strains. The identification and characterization of novel *Lactococcus* strains may reveal distinct attributes that have high technological potential for the dairy industry. This study aimed to characterize the technological and bacteriocinogenic potential of *L. lactis* subsp. *lactis* bv. diacetylactis strains obtained from dairy production systems. Twenty-three strains of *L. lactis* subsp. *lactis* isolated from cow, goat, and buffalo milks, cow's milk cream, artisanal cheeses from the Amazon region, artisanal cheeses from Marajó Island, forage peanut silages and grass silages were used in this study. The strains were identified at a biovar level (acetoin and dyacetil production, despite specific PCR assays), fingerprinted by rep-PCR and characterized for their technological potential: lactofermentation patterns, extracellular proteolytic activity, acidification capacity, and resistance to NaCl. Subsequently, the strains were subjected to PCR to detect bacteriocin-related genes (nisin, lacticins 481 and 3147, lactococcins A and 972) and further investigated by PCR and sequencing for the nisin operon. Cell free supernatants (CFS) of nisin-positive strains were tested by the spot-on-the lawn assay against a panel of 16 targets (*Listeria monocytogenes* - 4, *L.* innocua - 1, *Staphylococcus aureus* - 6, *Lactobacillus sakei* - 1, *L. lactis* - 4). Growth curves of 4 microbial targets (*L. monocytogenes* - 2, *S. aureus* - 2), with and without the CFS of nisin producers, were obtained by optical density (λ = 650 nm). Based on a culture collection of 23 *L. lactis* subsp. *lactis* strains, 15 presented molecular and phenotypical results that allowed them to be identified as *L. lactis* subsp. *lactis* bv. diacetylactis. The rep-PCR analysis showed 11 different genetic profiles with similarity of less than 90%, thus indicating a high level of diversity among the 15 isolates identified as *L. lactis* subsp. *lactis* bv. diacetylactis. The technological characterizations indicated that two strains did not present coagulation abilities and 13 were positive for extracellular proteolysis activity.

Many of the strains were shown to efficiently acidify skim milk though two strains showed high acidifying capacities, resulting in a pH decrease over 2 units after 24h. NaCl tolerance assay revealed that all these strains were able to grow at all tested concentrations (0, 2, 4, 6, 8 and 10%); nevertheless, strains obtained from non-dairy niches presented higher tolerance to NaCl when compared to the isolated obtained from dairy niches. Eight strains presented positive results only for *nisA*, and only one strain (SBR4) presented the full nisin operon, confirmed by sequencing as similar to nisin Z. Only SBR4 strain presented inhibitory activity by the spot-on-the lawn assay against the 16 microbial targets. Growth curves of selected targets confirmed the inhibitory activity of the SBR4 strain, especially against *S. aureus*, indicating its potential for nisin production. Therefore, these results suggest that the studied strains present important technological characteristics for the food industry and that their application is viable for the production of fermented foods. In view of the technological features and the ability to produce nisin of *L. lactis* subsp. *lactis* bv. diacetylactis SBR4, it can be used to constitute a starter or mixed culture, being a strain to be considered in the dairy industry as a bioconservative and biotechnological tool.

INTRODUÇÃO

Lactococcus lactis subsp. *lactis* pertence ao grupo das bactérias ácido láticas (BAL) e produzem ácido lático a partir do processo homofermentativo da glicose, sendo amplamente utilizado na indústria de alimentos, principalmente em derivados lácteos, e sua incorporação é reconhecida como segura. Atualmente, é crescente a demanda por novas cepas que possuam propriedades biotecnológicas e bacteriocinogênica para serem empregadas como cultura starter, contribuindo para o desenvolvimento de novos produtos, ou até mesmo, o aprimoramento das características dos produtos disponíveis comercialmente.

Dentro deste contexto destacam-se as cepas de *L. lactis* subsp. *lactis* bv. diacetylactis, que além de possuírem as propriedades habituais de uma cultura starter, são capazes de conferir características sensoriais pela conversão do citrato em diacetil e acetoína. A produção destes compostos aromáticos é relevante para o aperfeiçoamento dos atributos sensoriais em alimentos fermentados, especialmente os queijos.

Agregada as propriedades biotecnológicas, *L. lactis* subsp. *lactis* disponibiliza de propriedades bioconservantes que são caracterizadas pela produção de compostos antimicrobianos naturais, como as bacteriocinas. As bacteriocinas são peptídeos biologicamente ativos que apresentam atividade antimicrobiana contra micro-organismos deteriorantes e patógenos frequentemente associados aos alimentos. Dentre as bacteriocinas, destaca-se a nisina que é composta por 34 aminoácidos e pode ser produzida por linhagens de *L. lactis* subsp. *lactis*. A incorporação da nisina em alimentos é legalizada pelos órgãos competentes e geralmente ocorre pela adição do pré-peptídeo purificado ou pela fermentação *in situ* no alimento por cepas produtoras.

A caracterização de novas cepas de *L. lactis* subsp. *lactis* bv. diacetylactis é essencial para composição de culturas starters individuais ou mistas. Neste contexto, este estudo teve como objetivo identificar e caracterizar o potencial biotecnológico e bacteriocinogênico de cepas de *L. lactis* subsp. *lactis* bv. diacetylactis obtidas a partir do sistema de produção de leite, incluindo leite de diferentes espécies animais, queijos artesanais e silagens. O trabalho foi executado no Laboratório de Pesquisa em Leites e Derivados (InovaLeite) do Departamento de Tecnologia de Alimentos (DTA/UFV), no Laboratório de Inspeção de Produtos de Origem Animal (InsPOA) e Laboratório de Biologia Molecular (BioMol) do Departamento de Veterinária (DVT/UFV), ambos da Universidade Federal de Viçosa (UFV).

Esta dissertação consiste em dois capítulos: I. Identificação e caracterização tecnológicas de cepas de *L. lactis* subsp. *lactis* bv. diacetylactis, onde inicialmente foram identificadas as cepas por métodos moleculares e fenotípicos para o biovar diacetylactis e a sua similaridade genética. Posteriormente, as cepas foram submetidas a análises para definir as principais características tecnológicas que perfazem uma cultura starter, afim de selecionar cepas com propriedades biotecnológicas. II. Habilidade das cepas de *L. lactis* subsp. *lactis* bv. diacetylactis em produzir nisina. Nesta etapa as cepas foram submetidas a análises moleculares para expressão dos principais genes relacionaos a produção de bacteriocinas, com enfoque nos genes que compõem o operon de nisina e o sequenciamento de aminoácidos para o gene de expressão *nisA*. Subsequentemente, cepas com potencial bacteriocinogênico foram analisadas quanto ao seu espectro de ação frente a micro-organismos indicadores, verificando-se assim as propriedades bioconservadoras que podem vir a compor uma cultura starter para a indústria de alimentos.

REVISÃO BIBLIOGRÁFICA

1. Bactérias ácido láticas

As várias maneiras pelas quais os micro-organismos têm sido utilizados nos últimos anos para contribuir na indústria de alimentos nos processos tecnológicos e padrões de inocuidade e qualidade (Carr et al., 2002; Leroy & De Vuyst, 2004; Settanni & Corsetti; 2008; Kim et al., 2013; Martinez et al., 2013; Zannini et al., 2016; Kavitake et al., 2018), na saúde humana e animal com a aplicação de probióticos (Rashid & Sultana, 2016; Wang et al., 2016; O'Tolle et al., 2017; Robles-Vera et al., 2017; Kawai et al., 2018), na biotecnologia agrícola para o tratamento de sementes, tolerância a condições adversas e indução de respostas aumentadas a fertilizantes (Fox, 2015; Dhamale et al., 2015; Mitter et al., 2017), e no âmbito da proteção ambiental com a substituição de fertilizantes químicos e pesticidas tóxicos por fertilizantes orgânicos e agentes de controle biológico (Schoebitz et al., 2013; Baez-Bashan et al., 2014; Rogelio et al., 2016; Ramakrishna et al., 2019) fornecem um registro impressionante da importância microbiana. Neste contexto, as bactérias ácido láticas (BAL) despertam suma relevância tecnológica e têm sido tradicionalmente associadas à fermentação e preservação de alimentos, sendo nomeadas a partir de uma característica em comum: a produção de ácido lático como principal produto final da fermentação de carboidratos (Leroy & De Vuyst, 2004). A produção de ácido lático pode ocorrer por dois mecanismos: (1) homofermentativo, sendo o ácido lático o principal produto final; e (2) heterofermentativo, com a geração de diversos produtos finais, como dióxido de carbono, etanol ou acetato, além do ácido lático (Carr et al., 2002; Leroy & De Vuyst, 2004; Gänzle, 2015).

As BAL compreendem um grupo de 20 gêneros, e do ponto de vista prático para tecnologia de alimentos, os seguintes gêneros são considerados os principais: *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* e *Weissella* (Von Wright & Axelsson, 2012). O grupo das BAL é constituído de micro-organismos caracterizados por cocos ou bacilos Gram-positivos, ácidotolerantes e aerotolerantes, não formadores de esporos, geralmente não móveis (exceto *Vagococcus* spp. e algumas espécies de *Lactobacillus*), oxidase e catalase negativos (Leroy & De Vuyst, 2004; Von Wright & Axelsson, 2012).

As BAL desempenham um papel importante na fermentação e preservação de alimentos, seja como microbiota natural ou como cultura starter adicionada sob condições controladas, a fim de conferir características sensoriais e tecnológicas específicas (Settanni & Moschetti, 2010; Balciunas et al. 2013; Eş et al., 2018). O ácido láctico produzido pelas BAL é utilizado na indústria de alimentos devido ao seu papel na produção de iogurte e queijo. Na preparação de iogurtes é o principal produto da co-fermentação de *Streptococcus thermophilus* e *Lactobacillus bulgaricus*. Na fabricação de queijos, a diminuição do pH e consequente liberação de ácido lático desencadeia a agregação de micelas de caseína (Martinez et al., 2013). As propriedades higroscópicas e emulsionantes de alguns derivados do ácido lático, tais como ésteres de lactato, podem ser aplicados como emulsionantes e agentes de melhoria em produtos alimentícios (Gao et al., 2011). O sistema proteolítico das BAL na degradação da caseína fornece às células aminoácidos essenciais e contribuem para o desenvolvimento das propriedades sensoriais dos produtos lácteos fermentados (Savijoki et al., 2006). Além disso, destacam-se quanto ao aspecto de inocuidade dos alimentos, com a capacidade de inibir micro-organismos patogênicos e deteriorantes

frequentemente associados aos alimentos por meio da produção de substâncias antimicrobianas como ácido lático, ácido acético, ácido propiônico, dióxido de carbono, peróxido de hidrogênio, diacetil e, principalmente, bacteriocinas (Gálvez et al., 2007; Balciunas et al. 2013; Alvarez-Siero et al., 2016).

1.1. O gênero Lactococcus

As primeiras pesquisas sobre lactococos foram realizadas por Joseph Lister (Lister, 1873), na tentativa de provar a teoria de Pasteur. Utilizando leite fervido como meio nutriente nos experimentos, Lister obteve a primeira cultura bacteriana pura, nomeada *Bacterium lactis* (Josephsen & Jespersen, 2006; Tauber & Geis, 2006). Posteriormente, esta bactéria foi renomeada como *Streptococcus lactis* (Löhnis, 1909; Orla-Jensen, 1919; Josephsen & Jespersen, 2006; Tauber & Geis, 2006). Com base em estudos de hibridização de ácidos nucléicos, fisiológicos, imunológicos comparativos, lipídicos e lipoteicóicos, Schleifer et al. (1985) propuseram que *S. lactis* subsp. *lactis*, *S. lactis* subsp. *cremoris*, *Lactobacillus hordniae*, *Lactobacillus xylosus*, *S. garvieae*, *S. plantarum* e *S. raffinolactis* fossem classificados em um novo gênero: *Lactococcus*.

O gênero *Lactococcus* pertence à família *Streptococcaceae* e atualmente onze espécies de *Lactococcus* são reconhecidas: *L. lactis* (Lister, 1873), *L. raffinolactis* (Orla-Jensen, 1919), *L. garvieae*, *L. plantarum* (Collins et al., 1983), *L. piscium* (Williams et al., 1990), *L. chungangensis* (Cho et al., 2008), *L. fujiensis* (Cai et al., 2011), *L. taiwanensis* (Chen et al., 2013), *L. formosensis* (Chen et al., 2014), *L. hircilactis* e *L. laudensis* (Meucci et al., 2015). As bactérias deste gênero são caracterizadas como cocos Gram-positivos de 0,5 - 1 μm de diâmetro, podendo ocorrer de forma individual, em pares, cadeias curtas ou *clusters* irregulares. São anaeróbias

facultativas e mesofílicas com uma temperatura ótima de multiplicação a 30 °C, podendo sobreviver a 10 °C, mas não acima de 45 °C. Além disso, são neutrófilas com crescimento ótimo em meio com pH entre 6,3 a 6,9, podendo tolerar pH até 9,6 e são capazes de realizar a homofermentação da glicose produzindo ácido lático (Tauber & Geis, 2006; Von Wright, 2012). O teor molar de G+C (conteúdo de guanina-citosina) do DNA varia de 34 a 43% e o tamanho do genoma de diferentes *Lactococcus* foi estimado entre 2.300 e 2.600 kb (Schleifer et al., 1985; Tauber & Geis, 2006).

Lactococcus estão intimamente associados aos alimentos lácteos fermentados e as espécies de *L. lactis* e *L. raffinolactis* encontram-se registradas no Inventário de Culturas de Alimentos Microbianos da Federação Internacional de Laticínios (*Microbial Food Cultures*, *International Dairy Federation - IDF*) (Bourdichon et al., 2012). Entretanto, pouco se sabe sobre *L. raffinolactis*, a qual foi caracterizada como um constituinte de misturas starters mesofílicas complexas indefinidas e a sequência do genoma foi recentemente elucidada (Meslier et al., 2012; McAuliffe, 2018). *L. lactis* recebe atenção especial, por ser uma das BAL mais utilizadas na indústria de alimentos em todo o mundo (De Vos, 2011; Smid & Kleerebezem, 2014). *L. lactis* é dividido em quatro subespécies: *L. lactis* subsp. *lactis* (Lister, 1873), *L. lactis* subsp. *cremoris* (Orla-Jensen & Hansen, 1919), *L. lactis* subsp. *hordniae* (Latorre-Guzman et al., 1977) e *L. lactis* subsp. *tructae* (Pérez et al., 2011). Particular interesse é atribuído a cepas específicas de *L. lactis* subsp. *lactis* que são capazes de fermentar o citrato e produzir diacetil e acetoína, que são compostos aromáticos desejáveis em alguns tipos de queijos, e estas cepas são referidas como *L. lactis* subsp. *lactis* bv. diacetylactis (Kempler & McKay, 1981, Schleifer et al., 1985).

As cepas de *L. lactis* estão amplamente distribuídas em superfícies e em produtos de origem vegetal e animal, sendo o leite cru e o queijo os habitats mais

reconhecidos. Acredita-se que o habitat inicial de *L. lactis* seja material vegetal, devido ao uso de pastagens para alimentação ou o uso de outras plantas como forragens e cama para o gado, e isto tenha levado a contaminação do leite por essas bactérias que ao longo do tempo se adaptaram a esse novo ambiente (Kelly et al., 2010; Cavanagh et al., 2015). Com a habilidade de colonizar biótipos distintos e se adaptar a variados nichos do ambiente lácteo, *L. lactis* encontram-se presentes no leite cru de diferentes espécies de animais, como vaca, ovelha e cabra (Nomura et al., 2006; Giannino et al., 2009; Quigley et al., 2011; Perin & Nero, 2014; Zhang et al., 2017; Darwish et al., 2018), sendo comumente isoladas de queijos artesanais produzidos a partir de leite cru (Dal Bello et al., 2010; Dolci et al., 2010; Delcenserie et al., 2014; Pangallo et al., 2014; Martins et al., 2018). *L. lactis* também habita o trato digestório, respiratório superior e urogenital de humanos (Juge, 2012; Moonens & Remaut; 2017) e animais, como peixes (Balcázar et al., 2007; Balcázar et al., 2008; Itoi et al., 2009; Nguyen et al., 2017), além de nichos vegetais, tais como gramíneas e silagens (Nomura et al., 2006; Yang et al., 2010; Khota et al., 2016; Li et al., 2018).

Cepas de *L. lactis* subsp. *lactis* bv. diacetylactis são responsáveis por produzir compostos aromáticos e possuem mecanismos que estão envolvidos no transporte de genes de citrato (Figura 1) (Beimfohr et al., 1997, Passerini et al., 2013; Laroute et al., 2017). Neste mecanismo, o citrato é transportado pelo plasmídeo CitP citrato-permease e captado pela célula, para então ser clivado em acetato e oxaloacetato pela citrato liase (CitDEF) e suas proteínas auxiliares (CitC, CitX e CitG). O oxaloacetato é subsequentemente descarboxilado em piruvato pela oxaloacetato descarboxilase, CitM. A utilização de citrato leva ao acúmulo de piruvato que pode ser redirecionado para duas vias alternativas: (1) geração de acetato e/ou etanol e formiato; (2) geração

de diacetil e acetoína resultantes da condensação de duas moléculas de piruvato em α-acetolactato (Figura 1) (Drider et al., 2004; Laroute et al., 2017).

Na indústria de laticínios, para a rápida avaliação do potencial de uma cepa em produzir compostos aromáticos, a utilização de citrato é investigada pelo desenvolvimento das culturas alvo em meio de cultura Kempler e McKay (KMK) (Kempler & McKay, 1980; Kempler & McKay, 1981) ou produção de acetoína e diacetil usando a reação de Voges-Proskauer (King, 1948). Além destas abordagens fenotípicas, os protocolos de PCR também são adotados para amplificação de genes relacionada à via do citrato (Beimfohr et al., 1997, Passerini et al., 2013).

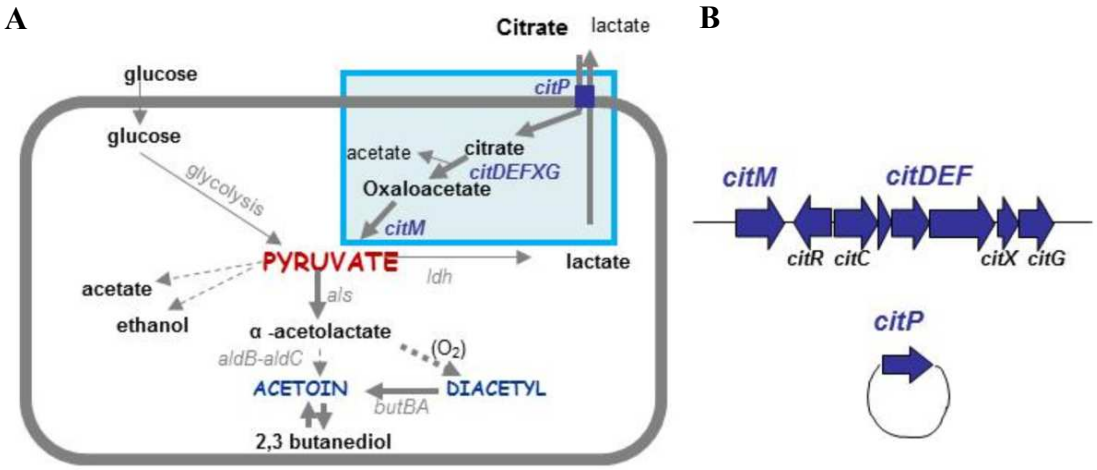

Figura 1. Mecanismo de produção de diacetil e acetoína. (A) Vias envolvidas no metabolismo do citrato e na produção de compostos aromáticos. (B) Genes de citrato e gene citP plasmidial envolvido no transporte de citrato.

Fonte: Laroute et al. (2017).

Além disso, outras cepas que utilizam o citrato podem produzir compostos aromáticos, mas através de um metabolismo mais lento pela fermentação da glicose e do redirecionamento do piruvato para os produtos finais da fermentação, resultando numa taxa muito baixa de compostos aromáticos, com diferentes modos de regulação

e dependentes de cada linhagem. Deste modo, considerar apenas as características genômicas não representa o potencial aromático de uma cepa. A representação das diferenças metabólicas é mais fácil pela análise dos fenótipos do que pelas análises moleculares para detectar sutis diferenças, provavelmente responsáveis pela heterogeneidade metabólica (Drider et al., 2004; Laroute et al., 2017).

1.2. Funcionalidades tecnológicas de Lactococcus lactis

A introdução de culturas starters puras de *L. lactis* na indústria láctea para o uso na fermentação de leite, nata e queijo originou-se no início da década de 1880 com o farmacêutico dinamarquês Christian D. A. Hansen, pioneiro no desenvolvimento de culturas starters comerciais com a produção de culturas líquidas e secas, sendo deste então aplicadas em diversos processos de fermentação de alimentos (Cogan, 1995; Josephsen & Jespersen, 2006). *L. lactis* possui a certificação GRAS (*Generally Recognized As Safe*) pela *Food and Drug Administration* (FDA) dos Estados Unidos da América, e preenche os critérios da *Qualified Presumption of Safety* (QPS) de acordo com a *European Food Safety Authority* (EFSA) (Cano-Garrido et al., 2015; Song et al., 2017). Atualmente *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis* e *L. lactis* subsp. *lactis* bv. diacetylactis são componentes essenciais de muitas culturas starters mesofílicas, individuais ou mistas, para a produção de diferentes tipos produtos de lácteos fermentados (Quadro 1).

Dentre as subespécies, *L. lactis* subsp. *lactis* e *L. lactis* subsp. *cremoris* apresentam maior interesse industrial para a constituição de uma cultura starter (Quadro 1) e uma das características essenciais é a produção de ácido lático a partir da lactose, influenciando, assim, as características sensoriais do produto fermentado final

(Lucey et al., 2003; Bachmann et al., 2009). Os baixos valores de pH (4,0 - 5,6) resultantes também previnem ou retardam o desenvolvimento de bactérias deteriorantes e patogênicas associadas aos alimentos (Gálvez et al., 2007; Balciunas et al. 2013). Adicionalmente, a coagulação do leite compreende a primeira etapa na fabricação de queijo através da desestabilização das micelas de caseína e quando atingido o ponto isoelétrico da caseína em pH 4,6, a caseína é precipitada conferindo a coalhada do leite para a produção de queijo cottage, quark, leite fermentado e iogurte (Lucey et al., 2003; Tauber & Geis, 2006).

Quadro 1. *L. lactis* como componentes em culturas starters para lácteos fermentados.

Tipo de produto	Composição da cultura starter
Tipo de queijo sem formação de olhaduras (Cheddar, Camembert, Tilsit)	*L. lactis* subsp. *cremoris*, 95 a 98%; *L. lactis* subsp. *lactis*, 2 a 5%
Queijo cottage, quark, leites fermentados, tipos de queijo com poucas ou pequenas olhaduras (ex. Edam)	*L. lactis* subsp. *cremoris*, 95%; *Leuconostoc mesenteroides* subsp. *cremoris*, 5%; ou *L. lactis* subsp. *cremoris*, 85 a 90%; *L. lactis* subsp. *lactis*, 3%; *L. mesenteroides* subsp. *cremoris*, 5%
Manteiga, leite fermentado, leitelho, tipos de queijo com olhaduras redondas (ex. Gouda)	*L. lactis* subsp. *cremoris*, 70 a 75%; *L. lactis* subsp. lactis bv. diacetylactis, 15 a 20%; *L. mesenteroides* subsp. *cremoris*, 2 a 5%
Taette (leite viscoso e azedo escandinavo)	*L. lactis* subsp. c*remoris*
Viili (leite viscoso finlandês)	*Oidium lactis*; *L. lactis* subsp. *cremoris*
Caseína	*L. lactis* subsp. *cremoris*
Kefir	*Lactobacillus kefir, Lb. kefiranofacians, L. lactis* subsp. *lactis*

Fonte: Tauber & Geis (2006).

Muitas cepas de *L. lactis* possuem uma protease associada à parede celular específica da β-caseína, juntamente com um complemento de peptidases. Este sistema proteolítico permite a quebra das caseínas e a liberação de aminoácidos favorecendo a

mutiplicação de *L. lactis* em leite, levando a precursores de aroma e a formação de sabor durante a fermentação láctea e a maturação do queijo (Liu et al., 2010; Tulini et al., 2016), sendo considerado o evento bioquímico mais importante na produção de queijo (Piraino et al., 2008). A atividade caseinolítica das proteases e peptidases de *L. lactis* tem uma grande influência na textura do queijo (Savijoki et al., 2006), no sabor do queijo, especialmente após a lise celular (Smit et al., 2005) e na liberação de peptídeos bioativos do leite (Leroy & De Vuyst, 2004).

Diacetil e acetoína são componentes essenciais para alguns produtos lácteos, sendo produzidos por *L. lactis* subsp. *lactis* bv. diacetylactis e responsáveis por confererir características sensoriais do produto com *flavor* cremoso e amanteigado (García-Quintáns et al., 2008; Laroute et al., 2017). As cepas de *L. lactis* subsp. *lactis* bv. diacetylactis podem converter o citrato em compostos aromáticos (C4) e dióxido de carbono que melhoram as características sensoriais de alimentos fermentados (García-Quintáns et al., 2008), como os queijos Camembert, Cheddar e Emmental (Curioni & Bosset, 2002, Leroy & De Vuyst, 2004). Para este fim, *L. lactis* subsp. *lactis* bv. diacetylactis é geralmente misturado com outra BAL durante a produção de queijo e a sua adição representa cerca de 20% da população total da cultura starter (Quadro 1) (Urbach, 1997, Carr et al., 2002, Smit et al., 2005).

O diacetil também é considerado um composto antimicrobiano que aumenta a inocuidade do produto final, agregando valor a incorporação da cultura starter (Deegan et al., 2006). A atividade antimicrobiana conferida a *L. lactis* é baseada na utilização de substrato, formação de ácido e diminuição do pH ou inibidores específicos, como reuterina, reutericiclina, ácidos graxos, peróxido e, principalmente, bacteriocinas (Cotter et al., 2005, Deegan et al., 2006; Gänzle, 2009).

2. Bacteriocinas

O primeiro relato de uma substância antimicrobiana foi realizado em 1925, quando André Gratia observou a capacidade inibitória da cepa *Escherichia coli* V frente outra cepa da mesma espécie, *E. coli* φ (Gratia, 1925; Collins et al., 2010). Nas décadas seguintes, várias substâncias antimicrobianas produzidas por bactérias entéricas foram identificadas e caracterizadas. Em 1946, Gratia juntamente com Pierre Fredericq, demonstrou a natureza proteica dessas substâncias e as chamou de "colicinas", como referência ao organismo produtor (Gratia & Fredericq, 1946; Collins et al., 2010). Mais tarde, a produção de substâncias semelhantes as colicinas por bactérias não entéricas foi descoberta (Florey et al., 1946; Cascales et al., 2007) e o termo geral "bacteriocina" foi utilizado para descrever substâncias semelhantes a proteínas com atividade restrita a espécies relacionadas (Jacob et al., 1953; Cascales et al., 2007).

As bacteriocinas são proteínas antimicrobianas que constituem um subgrupo heterólogo de peptídeos antimicrobianos sintetizados pelo ribossomo, liberados para o meio extracelular e biologicamente ativos, variando o seu espectro de atividade, modo de ação, massa molar, origem genética e propriedades bioquímicas (Chen & Hoover, 2003; Cotter et al., 2005; Cotter et al., 2013). A autoimunidade da cepa produtora pela sua própria bacteriocina é inibida por mecanismos de resistência (Kristiansen et al., 2016). A produção de bacteriocina pode ser induzida por co-cultivo com culturas vivas, células tratadas termicamente, sobrenadante ou purificada (Chanos & Mygind, 2016). Normalmente as bacteriocinas não são prejudiciais aos seres humanos, devido à sua alta especificidade para as membranas celulares bacterianas, sendo a bacteriocina citolisina uma exceção, produzida por *Enterococcus faecalis* (Cox et al., 2005).

De um modo geral, as bacteriocinas atuam tanto frente a bactérias Gram-positivas quanto Gram-negativas, oferecendo vantagem para a célula produtora sobre células não produtoras na competição de nutrientes em comum (Chen & Hoover, 2003; Cotter et al., 2005). Entretando, as bacteriocinas produzidas por BAL possuem maior espectro de ação contra as bactérias Gram-positivas. O espectro de inibição é geralmente bastante restrito, já que elas possuem atividade principalmente sobre bactérias intimamente relacionadas aos seus produtores, embora alguns peptídeos mostrem atividade antimicrobiana contra vários gêneros bacterianos (Cotter et al., 2005). A ação antimicrobiana pode ser de natureza bactericida ou bacteriostática e diversos parâmetros são considerados para a aplicação de bacteriocinas nos alimentos, como toxicidade, processamento, estabilidade, atividade de inibição de amplo espectro, efeito sobre as propriedades dos alimentos e uma compreensão completa de suas propriedades bioquímicas e genéticas (Cotter et al., 2005; Parada et al., 2007).

As bacteriocinas foram agrupadas em vários esquemas variando de I a V classes, considerando-se principalmente a estrutura primária, massa molar, estabilidade térmica e organização molecular (Klaenhammer, 1993; Nes et al., 1996; Cotter et al., 2005; Heng et al., 2007; Cotter et al., 2013). Apesar de não haver um consenso na literatura sobre a classificação das bacteriocinas, a classificação mais recente foi proposta por Cotter et al. (2013), onde as bacteriocinas produzidas por bactérias Gram-positivas são separadas das Gram-negativas. Deste modo, as bacteriocinas produzidas por bactéricas Gram-positivas foram divididas em dois grupos (classe I e II), englobando pequenos peptídeos sintetizados pelo ribossomo (Cotter et al., 2013).

O uso de BAL produtoras de bacteriocinas como agentes bioconservantes ou antibacterianos recebe cada vez mais atenção, visto que são substâncias naturalmente

produzidas e com aplicação em produtos lácteos para o controle de *Listeria monocytogenes* e *Staphylococcus* spp. (Dal Bello et al., 2012; Mendonça et al., 2012; Felicio et al., 2015; Perin et al., 2015; Cavicchioli et al., 2017; Wang et al., 2016; Mulkyte et al., 2017). *L. lactis* é capaz de produzir diferentes bacteriocinas, como por exemplo a nisina, lacticina 3147, lacticina 481 e diferentes variantes de lactococcinas (Alkhatib et al., 2012).

2.1. Nisina

Em 1928, Rogers observou a capacidade de certas cepas de *L. lactis* de produzir substâncias inibitórias em leite com atividade contra *Lactobacillus delbruecki* subsp. *bulgaricus* (anteriormente *Lactobacillus bulgaricus*) (Rogers, 1928). Posteriormente, em 1947, Mattick e Hirsh concentraram uma substância inibidora isolada de uma cepa de *L. lactis* subsp. *lactis*, denominado nisina (Mattick & Hirsh et al., 1947; Cotter et al., 2005). Hirsch e colaboradores (1951) descreveram a capacidade de inibição de anaeróbios formadores de esporos em queijo suíço por uma cepa de *L. lactis* produtora de nisina (Hirsch et al., 1951; Cotter et al., 2005). Deste modo, a nisina passou a ser usada como um aditivo alimentar para o controle de micro-organismos patogênicos e deteriorantes de alimentos, sendo inicialmente purificada e comercializada em 1953 na Inglaterra e, em 1969, foi considerada como GRAS para a utilização em produtos alimentícios pelo Comitê de Especialistas em Aditivos Alimentícios de Origem Animal (JECFA) da Organização das Nações Unidas para Agricultura e Alimentação (FAO) (Cotter et al., 2005; De Arauz et al., 2009). Em 1983 na União Européia, a nisina foi adicionada à lista de aditivos alimentares e, em 1988, o FDA autorizou seu uso em queijos processados (Cotter et al., 2005; Collins et al., 2010).

Embora a nisina seja a única bacteriocina aprovada pelo FDA para uso em alimentos, a pediocina obtida de cepas de *Pediococcus acidilactici*, *P. parvulus* e *Lactobacillus plantarum* WHE92, também tem sido empregada como conservante em alimentos industrializados (Ennahar et al., 1996; Simha et al., 2012; Wang & Wang, 2014; Fernandez et al., 2015; Silva et al., 2018). A pediocina produzida por *P. acidilactici* é utilizada como um componente para processos de fermentação e comercializada com a identificação ALTA 2431® (Microgard™, ALTA 2431, Quest Int., EUA) (Chen et al., 2004; Papagianni & Anastasiadou, 2009). No Brasil a comercialização da nisina é permitida como aditivo (ex. Nisaplin™, DuPont, EUA) e o seu uso é regulamentado pela Agência Nacional de Vigilância Sanitária (ANVISA). Entretanto, o uso da nisina é estrito como conservador na tecnologia de fabricação de queijo pasteurizado, queijo fundido e requeijão, com limite máximo de 12,5 mg/kg^{-1} (ANVISA, 1996).

A nisina é um polipeptídio pequeno (3,4 kDa), composto de 34 aminoácidos e se enquadra na classe I, definida como um lantibiótico, sendo a bacteriocina mais estudada e caracterizada para a via de biossíntese, genes do operon e modo de ação (Liu & Hansen, 1990; Alkhatib et al., 2012; Cotter et al., 2013). A nisina é produzida por muitas cepas de *L. lactis* e diversas variantes naturais de nisina foram descritas, com variação de aminoácidos e tamanho da molécula, contudo, todas apresentam atividade antimicrobiana muito similar. A variante nisina A foi a primeira a ser descrita (Rogers, 1928; Rogers & Whittier, 1928) e outras sete variantes naturais foram caracterizadas até o momento: nisina Z (Mulders et al., 1991), nisina Q (Zendo et al., 2003), nisina U e U2 (Wirawan et al., 2006), nisina F (Kwaadsteniet et al., 2008), nisina P (Zhang et al., 2012; Wu et al., 2014) e nisina H (O'Connor et al., 2015). As variantes de nisina A, Z, F e Q, são produzidas por cepas de *L. lactis* e a nisina U e

U2, produzidas por cepas de *Streptococcus* spp. (Piper et al., 2011). Dentre as variantes, comparando-se a estrutura primária de nisina A com a nisina Z, estas diferem apenas no aminoácido da posição 27 (histidina na nisina A e asparagina na nisina Z), enquanto nas demais variantes as modificações são maiores (Figura 2.A) (Mulders et al., 1991; Kwaadsteniet et al., 2008; Piper et al., 2011).

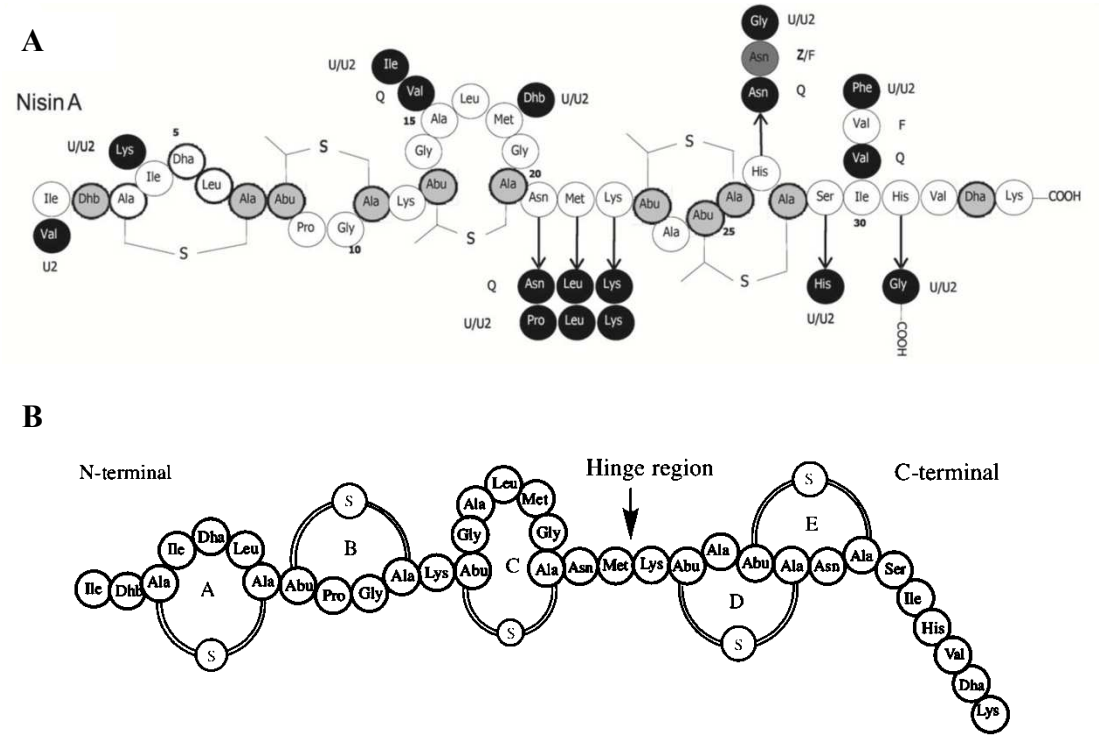

Figura 2. Estrutura da molécula de nisina A. (A) Comparação da estrurura da nisina A com as demais variantes naturais. (B) Estrutura da nisina A mostrando os anéis de lantionina A, B, C, D, E e a região de dobradiça flexível entre os anéis A-C e os anéis D-E.

Dha: diidroalanina; Dhb: diidrobutirina; Abu: ácido-2-aminobutírico; Ala-S-Ala: lantionina; Abu-S-Ala: β-metil-lantionina. Círculos pretos indicam diferenças de aminoácidos entre as variantes de nisina. Fonte: Piper et al. (2011); Breukink & Kruijff (2006).

A nisina é uma molécula anfipática, contém cinco anéis de lantionina/metil-lantionina (Figura 2.B), com uma parte N-terminal hidrofóbica e C-terminal hidrofílica (Field et al., 2015). A estrutura da nisina ativa compreende três partes: (1) uma região

N-terminal formada pelos anéis A e B (dois primeiros anéis); (2) uma região de dobradiça flexível; (3) e uma região C-terminal consistindo em anéis entrelaçados C, D e E seguidos de seis aminoácidos (últimos três anéis) (Figura 2.B) (Gross & Morell, 1970; Gross & Morell, 1971; Van De Ven et al., 1991). Os três primeiros anéis são importantes para ligação ao Lipídeo II, enquanto os dois últimos anéis são importantes para a formação de poros. A região da dobradiça flexível é crucial para a reorientação na membrana (Breukink et al., 1997; Breukink & Kruijff, 2006), e a presença desses anéis únicos na nisina é muito importante por sua atividade antimicrobiana e estabilidade (Breukink & Kruijff, 2006).

Quanto ao seu modo de ação, a nisina forma poros nas membranas de bactérias Gram-positivas, mediando o efluxo de íons, aminoácidos e ATP das células, levando à morte celular. O mecanismo inicia-se com a molécula alvo de nisina que é uma molécula precursora de síntese de parede celular lipídica II, que está ancorada na membrana citoplasmática (Wiedemann et al., 2001; Breukink & Kruijff, 2006). Esta âncora é conectada através de uma ponte éster a uma fração difosfato que transporta uma molécula de ácido N-acetilmuramico e a fixação de um pentapeptídeo completa a molécula lipídica II. Catalisados por uma reação de transglicosilação, as moléculas de ácidos N-acetilmuramicos se fundem em polímeros lineares formadores de lipídios II que são reticulados por uma reação de transpeptidação para formar o peptidoglicano maduro de bactérias Gram-positivas (Willey & Van Der Donk, 2007; De Kruijff et al., 2008). Em seguida, a nisina liga-se aos dois primeiros anéis (metil)-lantionina (A e B) na fração difosfato do lipídeo II (Hasper et al., 2004; Hsu et al., 2004) e evita a reação de transglicosilação, inibindo a síntese da parede celular (Wiedemann et al., 2001). Por fim, quando a concentração de complexos de nisina-lipídios II aumenta além de um certo limiar, é formado um complexo intermediário que induz a inserção do anel C de nisina na membrana citoplasmática. Isso resulta em um

complexo constituído por oito moléculas de nisina e quatro lipídeos, criando um poro com um diâmetro de 2 a 2,5 nm. A partir deste poro, são liberandos íons essenciais e pequenos nutrientes, causando um colapso do potencial da membrana e, posteriormente, conduzindo à morte celular (Hasper et al., 2004; Breukink & Kruijff, 2006).

O efeito inibitório da nisina é limitado, com espectro predominante sobre bactérias Gram-positivas, principalmente sobre BAL dos gêneros *Lactococcus* spp., *Enterococcus* spp e *Streptococcus* spp. (Chen & Hoover, 2003; Cotter et al., 2005; Piper et al., 2009). Pesquisas demonstram a inibição de bactérias patogênicas ou deteriorantes associadas aos alimentos, como *L. monocytogenes* (Benkerroum & Sandine, 1988; Pol & Smid, 1999; Kim et al., 2008; Bergholz et al., 2013; Campion et al., 2013; Alves et al., 2016) e *S. aureus* (Rilla et al., 2004; Millette et al., 2007; Pinto et al., 2011; Felicio et al., 2015; Perin et al., 2015; Wang et al., 2016), bem como células vegetativas ou esporos de *Bacillus* spp. (Pol & Smid, 1999; Ettayebi et al., 2000; Badaoui Najjar et al., 2007; Liu et al., 2015) e *Clostridium* spp. (Bartoloni et al., 2004). A nisina não apresenta atividade contra micro-organismos Gram-negativos, fungos e leveduras. Porém, se associada com agentes que modifiquem a permeabilidade da membrana externa, como EDTA, pode apresentar um potencial inibitório (Delves-Boughton, 1993; Cotter et al., 2013).

Em relação a expressão genética, a nisina é constituída em um conjunto de genes associados a um transposon conjugativo denominado *Tn5276* composto por onze genes organizados na ordem *nisABTCIPRKFEG*, onde cada qual exerce funções específicas: síntese, modificação, transporte, autoimunidade e regulação (Figura 3) (Lubelski et al., 2008; AlKhatib et al., 2012). O sistema de expressão inicia-se com a síntese ribossômica da nisina por um pré-peptídeo (pré-NisA) pelo gene *nisA* constituído por 57 aminoácidos, que é subdividido em uma sequência líder N-terminal (23 aminoácidos) que sinaliza o transporte para o complexo de modificação localizado na membrana plasmática da célula

produtora, e uma sequência C-terminal (34 resíduos de aminoácidos) (Figura 3) (Siegers et al., 1996; Van Den Hooven et al., 1997; AlKhatib et al., 2012).

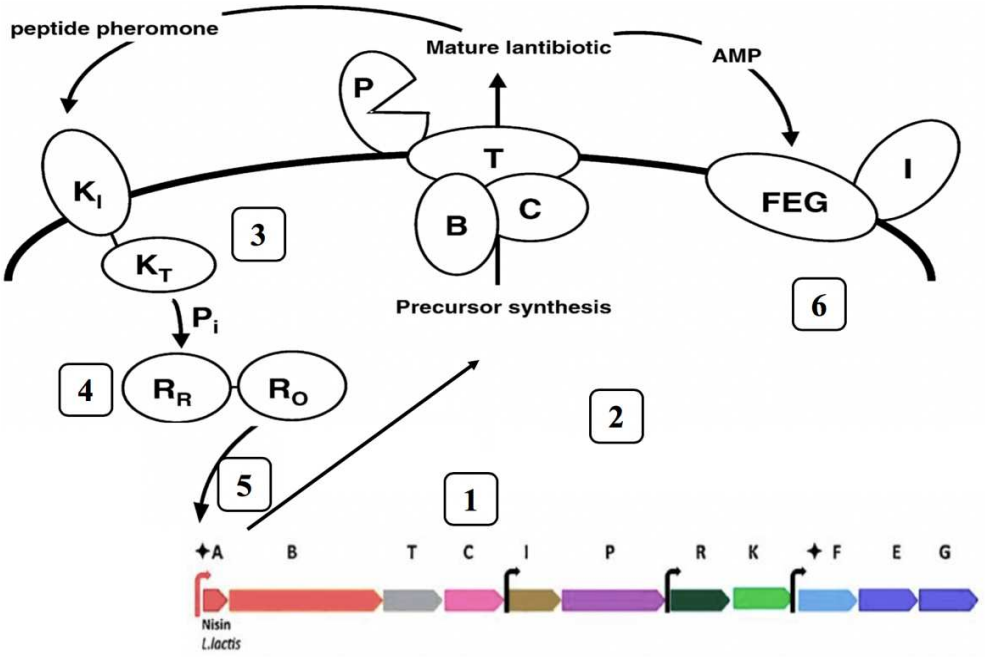

Figura 3. Visão geral do operon de nisina e modelo de biossíntese e regulação.

Promotor nisA (seta vermelha) e promotores *nisF*, *nisI* e *nisR* (preto), sendo os promotores marcados com ✦ controlados por um sistema de dois componentes *nisRK*, enquanto que a transcrição de *nisRK* e *nisI* (P) é constitutiva. (1) Formação de pré-péptido (pré-nisina); (2) O pré-peptídeo modificado por NisB e NisC, translocado através de um transportador ABC de NisT e processado por NisP, resultando na liberação de nisina madura; (3) A proteína quinase histidina (HPK) detecta a presença de bacteriocina e autofosforilados; (4) O grupo fosforil (Pi) é subsequentemente transferido para o regulador de resposta (RR); RR ativa a transcrição dos genes regulados; e (6) imunidade do produtor mediada por proteínas de imunidade, NisI e proteínas de transporte ABC de NisFEG. Fonte: adaptado de Kleerebezem (2004) e AlKhatib et al. (2012).

Após a síntese ribossomal por pré-NisA, a pré-nisina é modificada de forma pós-translacional através do mecanismo de modificação NisBC (Alkhatib et al., 2012), essencial para a maturação e biossíntese da nisina (Khusainov et al., 2011). NisB é uma enzima que desidrata resíduos de serinas e treoninas no pré-péptido (Sen et al., 1999) e NisC, uma metaloproteína dependente de zinco que cicliza resíduos desidratados para

resíduos de cisteína localizados na região C-terminal para formar anéis de metil-lantionina ou lantionina (Koponen et al., 2002; Alkhatib et al., 2012). Para que essas modificações biossintéticas ocorram, a pré-nisina totalmente modificada, mas inativa, chamada NisA madura (mNisA), é exportada para fora da membrana celular por NisT, um transportador ABC que forma um complexo associado à membrana com NisB e NisC (Qiao e Saris, 1996; Kuipers et al., 2004). Após a exportação, a mNisA é ativado proteoliticamente por clivagem do peptídeo líder e esse processo é realizado pela enzima serino-protease ancorada à membrana celular, NisP (Kuipers et al., 1993; Lagedroste et al., 2017), que separa da sequência peptídica líder, produzindo nisina madura constituída por 1 lantionina, 4 metilantioninas, 1 desidrobutirina, 2 desidroalaninas e 21 aminoácidos (Qiao & Saris, 1996; Plat et al., 2011).

A nisina atua como um sensor de quórum sensing e autorregula sua própria biossíntese pelo sistema de dois componentes formado por histidina-quinase (NisRK) que permite a autoindução do promotor de NisA (Kleerebezem, 2004; Chatterjee et al., 2005; Cheigh and Pyun, 2005). A nisina é reconhecida por NisK que está ligado à membrana citoplasmática e ocorre uma autofosforilação de resíduo de histidina de NisK. Esse alto potencial energético do grupo fosforil é transferido para um resíduo de aspartato conservado e regulador de resposta transcricional, NisR. Após a ativação, NisR liga-se ao promotor NisA, estimulando a expressão dos genes do operon de nisina (Kuipers et al., 1995; De Ruyter et al., 1996). Estudos demonstram que os dois primeiros anéis de nisina são essenciais para o mecanismo de regulação, enquanto o anel C de nisina é importante para a indução da expressão, e os dois últimos anéis não têm influência específica sobre a expressão (Chan et al., 1989; Kuipers et al., 1995).

O operon da nisina contém quatro genes que codificam proteínas de autoimunidade, atuando em um sistema cooperativo: *nisF*, *nisE* e *nisG* codificam um

transportador ABC com função imune contra nisina (Alkhatib et al., 2014) e *nisI*, que codifica a lipoproteína NisI de 245 aminoácidos com sequência sinal que está envolvida na autoproteção (Qiao et al., 1995; Siegers & Entian, 1995). A imunidade total é obtida apenas quando *nisI* e *nisFEG* são expressos (Stein et al., 2003), mas os mecanismos pelos quais ambos participam na proteção contra a nisina ainda são desconhecidos.

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OBJETIVOS

Objetivo geral

Identificar cepas de *L. lactis* subsp. *lactis* bv. diacetylactis e caracterizar os aspectos ligados ao potencial tecnológico e a habilidade de produzir nisina.

Objetivos específicos

- Identificar a coleção de culturas de *L. lactis* subsp. *lactis* a nível de biovariedade através de técnicas moleculares e fenotípicas;
- Caracterizar o potencial tecnológico de cepas de *L. lactis* subsp. *lactis* bv. diacetylactis;
- Avaliar o potencial bacteriocinogênico das cepas;
- Realizar a identificação molecular dos genes relacionados a produção de nisina;
- Realizar o sequenciamento de amino ácidos do gene de expressão de nisina;
- Avaliar o espectro de atividade antimicrobiana das cepas produtoras de nisina frente a diferentes micro-organismos indicadores.

CAPÍTULO 1 – Technological properties of *Lactococcus lactis* subsp. *lactis* bv. diacetylactis obtained from dairy and non-dairy niches

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Manuscript prepared and submitted to *Brazilian Journal of Microbiology* (IF 1.810)

Title page

Technological properties of *Lactococcus lactis* subsp. *lactis* bv. diacetylactis obtained from dairy and non-dairy niches

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ABSTRACT

Lactococcus lactis subsp. *lactis* bv. diacetylactis strains are used in the dairy industry to generate acetoin and diacetyl, which impart high levels of buttery flavor notes. The identification and characterization of new lactococcal strains from dairy and non-dairy niches may reveal distinct attributes that have great technological potential for the dairy industry. Twenty-three strains of *L. lactis* subsp. *lactis* were isolated from cow, goat, and buffalo milks, cow's milk cream, artisanal cheeses from the Amazon region, artisanal cheeses from Marajó Island, forage peanut silages and grass silages. The strains were identified at a biovar level, fingerprinted by rep-PCR and characterized for their technological potential. Based on a culture collection of 23 *L. lactis* isolates, 15 of the specimens collected presented molecular and phenotypical (diacetyl and citrate) results that allowed them to be identified as *L. lactis* subsp. *lactis* bv. diacetylactis. The rep-PCR fingerprinting analysis determined that the 15 isolates presented 11 different genetic profiles with lower than 90% of similarity, thus indicating a high level of diversity among them. The technological characterizations indicated that two isolates did not present coagulation abilities and 13 were positive for extracellular proteolysis activity. Many of the isolates were shown to efficiently acidify skim milk though two isolates showed high acidifying capacities, resulting in a pH decrease over 2 pH units after 24h. NaCl tolerance assay revealed that all these isolates were able to grow even at the highest concentrations assessed. However, strains from non-dairy niches showed an even higher tolerance to NaCl (10%). These results indicate that the studied strains present important technological characteristics for the food industry and that their application is viable for the production of fermented foods.

Key-Words: lactic acid bacteria; diacetyl; starter culture; technological potential

INTRODUCTION

Lactococcus lactis is a lactic acid bacteria (LAB) of particular interest in the dairy industry due to its technological potential as starter culture; it is widely employed to produce fermented milks and ripened cheeses (Leroy and De Vuyst, 2004). Among the *L. lactis* subspecies, *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* are the two most often associate with the fermentation processes. These have therefore been the focus of many studies that examine the characterization of promising and new starter cultures (Schleifer et al., 1985, Pérez et al., 2011, Laroute et al., 2017).

Particular interest has been paid to the specific strains of *L. lactis* subsp. *lactis* that are capable of fermenting citrate and producing diacetyl and acetoin, the desirable flavor compounds in specific ripened cheeses; these strains are referred as *L. lactis* subsp. *lactis* bv. diacetylactis (Kempler and McKay, 1981, Schleifer et al., 1985). *L. lactis* subsp. *lactis* bv. diacetylactis strains can convert citrate to aroma compounds (C4) and carbon dioxide which improve the organoleptic characteristics of fermented foods (García-Quintáns et al., 2008). Moreover, diacetyl is an essential component of many dairy products, since it lends a creamy and buttery aroma when present at low concentrations. It contributes to the traditional attributes of certain specific dairy products, such as Camembert, Cheddar and Emmental cheeses (Curioni and Bosset, 2002, Leroy and De Vuyst, 2004). To this end, *L. lactis* subsp. *lactis* bv. diacetylactis is generally mixed with other LAB during cheese production; the addition generally accounts for 20% of the total population of the added starter culture (Urbach, 1997, Carr et al., 2002, Smit et al., 2005). In addition to these organoleptic benefits, diacetyl is also considered to be an antimicrobial compound that enhances product safety when used as part of a starter culture (Cotter et al., 2013).

Laroute et al. (2017) explain that aroma -producing strains with potential use in the dairy industry must be screened to determine their citrate-depleting potential. This characteristic can be assessed by studying the strains' growth in a Kempler and McKay (KMK) medium, followed by a study of their acetoin and diacetyl production using the Voges-Proskauer reaction. In addition to this phenotypic approach, PCR protocols must also be adopted to focus on gene amplification related to the citrate pathway and to characterize the mosaic structure of the histidine biosynthesis operon (Beimfohr et al., 1997, Passerini et al., 2013).

This study aimed to present a comprehensive characterization of *L. lactis* subsp. *lactis* strains obtained from dairy environment in order to identify bv. diacetylactis strains. The technological properties of the identified bv. diacetylactis strains have also been characterized in order to determine promising and potential starter cultures that could be used in the dairy industry in the future.

MATERIAL AND METHODS

Strains

A total of 23 *L. lactis* subsp. *lactis* isolates were included in this study. The isolates were obtained from the bacteria culture collection at InovaLeite (Laboratory of Milk and Dairy Products, Universidade Federal de Viçosa). These have been isolated from different ecosystems, including dairy production environments (grass and peanut silages, raw milk from different animals) and artisanal cheese production sites in the Amazon region and on Marajó Island. All isolates were previously identified as *L. lactis* via regional sequencing of the 16S rRNA, and further subspecies-specific PCR assays which targeted *L. lactis* subsp. *lactis*. *L. lactis* subsp. *lactis* bv. diacetylactis

ATCC 13675 was used as a positive control and *L. lactis* subsp. *cremoris* ATCC 19257 was used as negative control in the molecular assays (Beimfohr et al., 1997). *Pseudomonas fluorescens* 07A (Alves et al., 2016) was used as positive control in the extracellular proteolytic activity assay.

Identification of L. lactis subsp. lactis bv. diacetylactis

The selected strains were cultured in de Man, Rogosa and Sharpe (MRS) broth (Oxoid Ltd., Basingstoke, England) at 30 °C for 18 h. One mL aliquots of the cultures were centrifuged at 14,000 × *g* for 2 min, and the cell pellets DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). A PCR assay was conducted to identify the isolates from bv. diacetylactis according Beimfohr et al. (1997), using primers Lhis5F (5'-CTTCGTTATGATTTTACA-3') and Lhis6R (5'-AATATCAACAATTCCATG-3'). PCR conditions were: (1) 2 min at 93 °C, (2) 30 cycles of 30 s at 94 °C; 90 s at 46 °C and 2 min at 72 °C, and (3) final extension of 5 min at 72 °C. The obtained PCR products were electrophoresed on 1.5% agarose gels (w/v), stained using GelRed (Biotium Inc., Hayward, CA, USA) and visualized using a transilluminator LPIX (Loccus Biotecnologia, São Paulo, SP, Brazil). The presence of a 934 bp band was considered to indicate a positive result.

Isolates were also characterized by their ability to ferment citrate and produce diacetyl (King, 1948, Kempler and McKay, 1980). Aliquots of the obtained cultures were streaked onto Kempler and McKay agar and incubated at 30 °C for 48 h; isolates that presented blue colonies were categorized as citrate-fermenting (Passerini et al., 2013). To determine diacetyl production, aliquots of the cultures were transferred to sterile skim milk (10% w/v, Nestlé, São Paulo, SP, Brazil) and incubated at 30 °C for 24 h; then, 1 mL aliquots of the obtained cultures in milk were added to 0.5 mL of α-naphthol

(1% w/v) and KOH (16% w/v) and incubated at 30 °C for 10 min; diacetyl production was indicated by the formation of a red ring at the top of the tubes (King, 1948).

Isolates that demonstrated PCR amplification, diacetyl production, and citrate fermentation were considered to be *L. lactis* subsp. *lactis* bv. diacetylactis, and then subjected to fingerprinting and characterization of their technological potential, as described below.

rep-PCR fingerprinting

Rep-PCR was performed according Dal Bello et al. (2010) 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) and recorded using a transilluminator LPIX (Loccus). The fingerprints were analyzed using BioNumerics 4.6 (Applied Maths, Kortrijk, Belgium). The similarities among profiles were calculated using the Pearson correlation, and a dendrogram was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

Technological properties of L. lactis subsp. lactis bv. diacetylactis

Isolates identified as *L. lactis* subsp. *lactis* bv. diacetylactis were subjected to phenotypic assays to assess their technological properties. All assays described below were conducted in three independent repetitions.

Lactofermentation patterns were assessed by inoculating 1 mL aliquots of the isolate cultures into 10 mL of skim milk (10% w/v, Nestlé) which was then incubated at 30 °C for 24 h. Based on the formed clot characteristics, the lactofermentation patterns were described using an empirical analysis and classified as: uniform, uniform with presence of serum, uniform and fragile (appearance), broken with presence of serum and absence of clot.

Extracellular proteolytic activity was determined according to the protocol proposed by Franciosi et al. (2009). Two µL aliquots of bacterial cultures were spotted onto the surface of a Plate Count Agar (PCA, HiMedia, Mumbai, MH, India) enhanced with skim milk (10% w/v, Nestlé). These were incubated at 30 °C for 4 days. Proteolytic activity was indicated by a clear zone around the formed colonies.

The acidification capacity of *L. lactis* subsp. *lactis* bv. diacetylactis isolates was assessed by adding culture aliquots to skim milk(10% w/v, Nestlé), which was then incubated at 30 °C for 24 h. pH values were measured at the time of inoculation (T0), then after 6 (T6), 12 (T12) and 24 h (T24) of incubation using a digital pHmeter (Hanna Instruments, São Paulo, SP, Brazil). Based on the cultures' abilities to reduce milk pH after 24 h, the tested isolates were divided into 3 main groups: (I) high acidifying isolates with a pH decrease over 2 pH units; (II) medium acidifying isolates with a pH drop of 1.5 to 2.0 pH units; (III) low acidifying isolates with a pH decrease lower than 1.5 pH units (Psoni et al., 2007).

Finally, resistance to NaCl was assessed as described by Dal Bello et al. (2012). Two µL aliquots of the selected isolates were transferred to 188 µL of MRS broth (Oxoid) prepared with different concentrations of NaCl (0, 2, 4, 6, 8 and 10%, w/v). These had been previously distributed into 96-well microtiter plates. The experiment was conducted in duplicate. In each prepared microtiter plate, 2 blank wells with only 190

μL of MRS broth (Oxoid) were prepared for each concentration of NaCl. The microplates were then incubated in Multiskan^{TM} GO Microplate Spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA) at 30 °C, agitated for 24 h and measured for optical density (OD) of cultures every 30 min (λ = 650 nm). OD readings were plotted in graphics in order to demonstrate the growth curves of *L. lactis* subsp. *lactis* bv. diacetylactis isolates.

RESULTS AND DISCUSSION

Of the 23 tested isolates of *L. lactis* subsp. *lactis*, 17 were found to be amplified PCR products with 934 bp (Table 1), typical of bv. diacetylactis according Beimfohr et al. (1997). Based on the adopted phenotypical assays, 17 isolates were shown to be able to able to ferment citrate, and 20 were able to produce diacetyl (Table 1). BUF1 showed a typical PCR result for bv. diacetylactis but it was not able to ferment citrate or produce diacetyl, whereas SBR3 was only unable to produce diacetyl. Conversely, LVTC8MRS and Q1C4 did not present positive PCR results, although they were able to ferment citrate and produce diacetyl. Q13C4 was able to produce diacetyl even though it did not present a typical PCR result for bv. diacetylactis (Table 1).

Even though identifying bv. diacetylactis isolates usually only calls for phenotypical assays (Nomura et al., 2006, Kahala et al., 2008, Cavanagh et al., 2015), we have examined the molecular assays associated with the ability to produce diacetyl and ferment citrate in this study. Based on these criteria, 15 isolates were identified as belonging to bv. diacetylactis (Table 1). According to Siezen et al. (2011), strains that can utilize citrate and stimulate the production of acetoin and diacetyl present an interesting phenotypic trait for potential use in the dairy industry Therefore the ability

to produce diacetyl must be considered as the main criterion when identifying bv. diacetylactis isolates. The adopted PCR approach is both convenient and reliable for identifying *L. lactis* subsp. *lactis* bv. diacetylactis, when based on specific primers that target the mosaic structure of the *L. lactis* histidine biosynthesis operon (Beimfohr et al., 1997).

Diacetyl production is a commonly described trait for *L. lactis* subsp. *lactis* (Franciosi et al., 2009, Dal Bello et al., 2012, Domingos-Lopes et al., 2017, Perin et al., 2017). It is important to note that the acetoin/diacetyl pathway is essential for the generation of the aroma compounds hat result in a buttery characteristic in cheeses (Starrenburg and Hugenholtz, 1991). Diacetyl production is strain-dependent, since not all LAB are able to metabolize citrate. Moreover, this behavior may differ between species and subspecies. Diacetyl is a volatile compound generated as the final product when citrate converts to pyruvate. It imparts a buttery aroma to fermented dairy products. It is associated with *L. lactis* subsp. *lactis* bv. diacetylactis (Kempler and McKay, 1981, Smit et al., 2005).

For rep-PCR fingerprinting analysis, strains identified as *L. lactis* subsp. *lactis* bv. diacetylactis were selected and grouped according to a 90% of similarity or above (Figure 1). The 15 isolates were grouped in 11 profiles and the groups formed showed low homology to each other, indicating a high diversity among the strains of *L. lactis* subsp. *lactis* bv. diacetylactis. There were no profiles with more than two similar isolates and the maximum homology was found between isolates Q1C5 and Q1C10 which presented a 94.6% homology. Both of these were isolated from artisanal cheeses from the Amazon region. The LVA2.1 and LCA5 lines of cow and goat milk, respectively, presented a 91.9% similarity. The LVA2VACA (cow milk) and LCA2 (goat milk) isolates presented a 91.5% similarity. Q5C6 isolate, obtained from artisanal

cheese from the Amazon region showed low similarity (41.1%) in relation to the other milk strains, it may therefore present interesting phenotypic characteristics for industrial applications. The Q15C3 strain taken from an artisanal Marajó Island cheese showed 88.8% homology with the isolated Q4C8 strains of artisanal cheese from the Amazon region and indicated that there may be high levels of similarity between artisanal cheese isolates from different regions. The dairy-origin strains were expected to demonstrate greater similarity than plant-origin strains, because there are gene losses, mutations and acquisitions that allow the strains to adapt to new habitats which occur during adaptation (Siezen et al., 2011, Laroute et al., 2017). Regarding the three plant-origin isolates, The SBR1 and SBR4 strains from grass silage were grouped together, with an 80.3% similarity, while the peanut silage strain SAM12 presented low similarity to all the other isolates (Figure 1). Because they are so genetically different as plant-origin isolates, the SAM12 strain and the Q5C6 isolate may also present genotypic characteristics that are unique and of interest to the food industry.

The technological properties of the selected *L. lactis* subsp. *lactis* bv. diacetylactis isolates are presented in Table 2 (lacto-fermentation, extracellular proteolytic activity, and acid production) and in Figure 2 (growth at different NaCl concentrations). Only 2 isolates did not present coagulation abilities (Table 2); the absence of clot formation was observed in isolates obtained from non-lactic niches, except for SBR4 (Tables 1 and 2). Milk coagulation comprises the first stage in cheese manufacturing through the destabilization of casein micelles, which brings them together to form cheese curds. This lactofermenting stage can occur through coagulant enzyme or specific lactic culture activity and results in the metabolic release of lactic acid. The lactic acid reduces pH and consequently inhibits the development of microorganisms while increasing coagulant activity through serum expulsion (Lucey et al., 2003). The

obtained results indicate the potential use of the characterized isolates as coagulant agents during cheese production.

Extracellular proteolysis is considered as the most important biochemical event in cheese production because it leads to the development substances that are either important for flavor or act as aroma precursors (Piraino et al., 2008). In this study, 13 isolates were shown to be able to break down casein (Table 2). Again, isolates from non-dairy niches did not present this characteristic, except for the SBR4 strain (Tables 1 and 2). These results were similar to those produced by several *L. lactis* subsp. *lactis* strains isolated from dairy niches (Franciosi et al., 2009, Dal Bello et al., 2012, Perin et al., 2017). Nomura et al. (2006) described the proteolytic activity in *L. lactis* subsp. *lactis* strains from fermented vegetables and cheese, though no proteolytic activity was observed with the bv. diacetylactis strains. Herreros et al. (2003) analyzed *L. lactis* subsp. *lactis* and *L. lactis* subsp. *lactis* bv. diacetylactis isolated from Armada cheese (a Spanish goat's milk cheese). In their research, the bv. diacetylactis strains showed low proteolytic activity.

Extracellular proteolytic activity is an essential property for starter cultures. The proteolytic system in LAB favors optimal growth in milk due to the consumption of caseins and peptides, which leads to the development of adequate cheese texture, aroma precursors and flavor formation during dairy fermentation and cheese maturation (Liu et al., 2010; Tulini et al., 2016). Only one isolate from a non-dairy niche demonstrated this characteristic (SBR4, Table 2). Nevertheless, it is of great importance to assure a well-balanced breakdown of caseins by strains, because excessive proteolysis may develop such undesirable attributes in cheese as low viscosity and high bitterness (Visser, 1993; González et al., 2010).

Many bv. diacetylactis isolates were shown to be efficient in acidifying skim milk by reducing the pH during the incubation period of 24 h at 30 °C (Table 2). A rapid drop in pH is crucial during cheese production because it contributes to changes in cheese texture and helps control the development of undesirable microorganisms. Two out of the 15 lactococcal strains lowered milk pH by more than 1.25 pH units after 12 h (Q1C5 and Q4C8), indicating fast acidification capabilities (Table 2). Studies have shown that most strains of *L. lactis* are initially slow in acid production (Franciosi et al., 2009, Morandi et al., 2011, Dal Bello et al., 2012) and the isolates in the present study demonstrated high levels of acidification of milk only after 24 h (Table 2). Herreros et al. (2003) presented a similar acidification capacity of *L. lactis* subsp. *lactis* and bv. diacetylactis strains, that showed a pH drop to 4.2 after 24h of incubation.

L. lactis subsp. *lactis* bv. diacetylactis is used globally as a starter LAB and represents a domesticated microorganism in which the citrate and acetoin/diacetyl pathways are coordinated and expressed at low pH (Zuljan et al., 2016). Dairy-related isolates were classified in groups I, II and III based on their abilities to reduce the milk pH after 24 h. Non-dairy strains only appear in group III (Tables 1 and 2). This finding demonstrates the dairy isolates' adaptability in regards to milk because the acidification they allow enhances their potential as starter cultures in dairy matrixes. Cavanagh et al. (2015) showed that non-dairy strains of *L. lactis* would be unsuitable for use as starters, as they are unable to reach the desired pH. However, because these strains are capable of growth in milk without the use of supplements, they were used as adjuncts in our study. Although most of the tested isolates were initially slow acidifiers, most acid production increased later. After 24 hours, 33.33% of the isolates could be grouped in class I and II (Table 2). Class III strains with low acidification profiles may contain technical characteristics suitable for adjunct cultures to be used

in the production of certain types of cheese (Gobbetti et al., 2015, Domingos-Lopes et al., 2017). Therefore, the variable acidifying activity indicates that this feature is strain-dependent. According to Wouters et al. (2002), it is common for wild lactococci to be less acidifying than commercial strains. According to our rep-PCR fingerprinting analysis, isolates Q1C5 and Q1C10 showed the greatest similarity (94.6%, Figure 1). However, these isolates have very different acidification capacities: Q1C5 demonstrated high acidification with a final pH of 4.45 but isolate Q1C10 presented low acidification with a final pH of 5.79. This demonstrates how genetically similar isolates may present different technological characteristics (Table 2).

The NaCl tolerance assay revealed that all the bv. diacetylactis isolates were able to grow even at the highest concentrations assessed (Figure 2). All isolates showed excellent growth in NaCl concentrations up to 4%. Six strains showed low growth at 8% and 10% NaCl. Strains from non-dairy niches showed a high tolerance up to 10% and with similar behavior at all concentrations. However, the SAM12 strain demonstrated low similarity with all the other strains (Figure 1) in rep-PCR fingerprinting analysis. Similar results were observed for the isolates LVA2.1, LVA2VACA and LCA1 which demonstrated similar compositions during the salt test (Figures 1 and 2). Also rep-PCR revealed the similarity of LVA2.1 and LCA1 to be 57% and LCA2VACA's similarity quotient was 11.2% (Figure 1). In an 8% salt environment, Q1C5 and Q1C10, two strains that showed the highest similarity in rep-PCR, presented distinct behaviors where Q1C5 was viable (Figures 1 and 2). The ability of starter strains to adapt and survive in various salt concentrations (including high concentrations) is extremely important during cheese production. Some *L. lactis* subsp. *lactis* tested by Perin et al. (2017) were able to grow in 10% NaCl, while Dal Bello et al. (2012) were unable to identify growth of LAB strains inoculated in culture

media added with NaCl at concentrations higher than 6%. Nomura et al. (2006) reported that all of the *L. lactis* subsp. *lactis* strains grew well in a 6.5% NaCl medium, while five of eight bv. diacetylactis strains from milk and five of eight *cremoris* strains failed to grow. The salt tolerance of certain *Lactococcus* isolates taken from cheese may also reflect an adaptation to the cheese environment (2 to 10% NaCl in moisture for different Caciocavallo varieties) and strains isolated from cheeses with the lowest salt content have low salt tolerance (Piraino et al., 2008).

CONCLUSIONS

The present study demonstrated that similar isolates may present broadly disparate results. The technological characterization of *L. lactis* subsp. *lactis* bv. diacetylactis isolates obtained from milk and non-dairy niches showed favorable technological potential and indicate possible use in as starter cultures in the dairy industry. However, the technological application of these depends on the functions that the microorganisms will exert over the final product, as well as on the product to which it will be added. In addition, some of the isolates could be used to constitute a mixed culture whose cheese-making characteristics can be evaluated in the future.

ACKNOWLEDGMENTS

CNPq, CAPES (financial code 001) and FAPEMIG.

CIRM-BIA for the concession of references cultures.

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Table 1. Molecular and phenotypic characterization of *L. lactis* subsp. *lactis* isolates targeting the identification of *L. lactis* subsp. *lactis* bv. diacetylactis.

isolate	origin	PCR[1]	citrate	diacetyl
LVA2.1	cow milk	+	+	+
LVA2.2	cow milk	+	+	+
LVA2VACA	cow milk	+	+	+
LCA1	goat milk	+	+	+
LCA2	goat milk	+	+	+
LCA4	goat milk	-	-	-
LCA5	goat milk	+	+	+
BUF1	buffalo milk	+	-	-
LVTC8MRS	cream milk (cow)	-	+	+
Q1C2	artisanal cheese (AM)[2]	-	-	+
Q1C4	artisanal cheese (AM)	-	+	+
Q1C5	artisanal cheese (AM)	+	+	+
Q1C7	artisanal cheese (AM)	+	+	+
Q1C10	artisanal cheese (AM)	+	+	+
Q4C8	artisanal cheese (AM)	+	+	+
Q5C6	artisanal cheese (AM)	+	+	+
Q6C2	artisanal cheese (AM)	-	-	-
Q13C4	artisanal cheese (Marajó)[3]	-	-	+
Q15C3	artisanal cheese (Marajó)	+	+	+
SAM12	peanut silage	+	+	+
SBR1	grass silage	+	+	+
SBR3	grass silage	+	-	+
SBR4	grass silage	+	+	+

[1] PCR product of 934 bp was indicative as typical of *L. lactis* subsp. *lactis* bv. diacetylactis (Beimfohr et al., 1997); [2] artisanal cheese obtained from the Amazonian region, Brazil; [3] artisanal cheese obtained from the Marajó Island, Northern region of Brazil. + = positive; - = negative.

Table 2. Technological properties of isolates identified as *L. lactis* subsp. *lactis* bv. diacetylactis.

isolate	lactofermentation	extracellular proteolytic activity	pH				
			T0	T6	T12	T24	Group
LVA2.1	uniform	+	6.7	6.4	6.03	6.01	III
LVA2.2	uniform	+	6.7	6.19	5.96	5.78	III
LVA2VACA	broken with serum	+	6.7	5.98	5.53	4.96	II
LCA1	uniform	+	6.7	6.26	6.06	5.17	II
LCA2	uniform	+	6.7	6.37	5.8	5.66	III
LCA5	uniform	+	6.7	5.99	5.64	5.38	III
Q1C5	uniform with serum	+	6.7	5.88	5.45	4.54	I
Q1C7	uniform	+	6.7	6.28	6.17	6.13	III
Q1C10	uniform	+	6.7	6.25	6.07	5.79	III
Q4C8	uniform with serum	+	6.7	5.91	5.37	4.66	I
Q5C6	uniform with serum	+	6.7	6.23	5.87	5.45	III
Q15C3	broken with serum	+	6.7	6.13	5.61	4.91	II
SAM12	absence of clot	-	6.7	6.33	6.22	5.91	III
SBR1	absence of clot	-	6.7	6.33	6.22	5.91	III
SBR4	uniform and fragile	+	6.7	6.33	6.12	6.04	III

+ = positive; - = negative

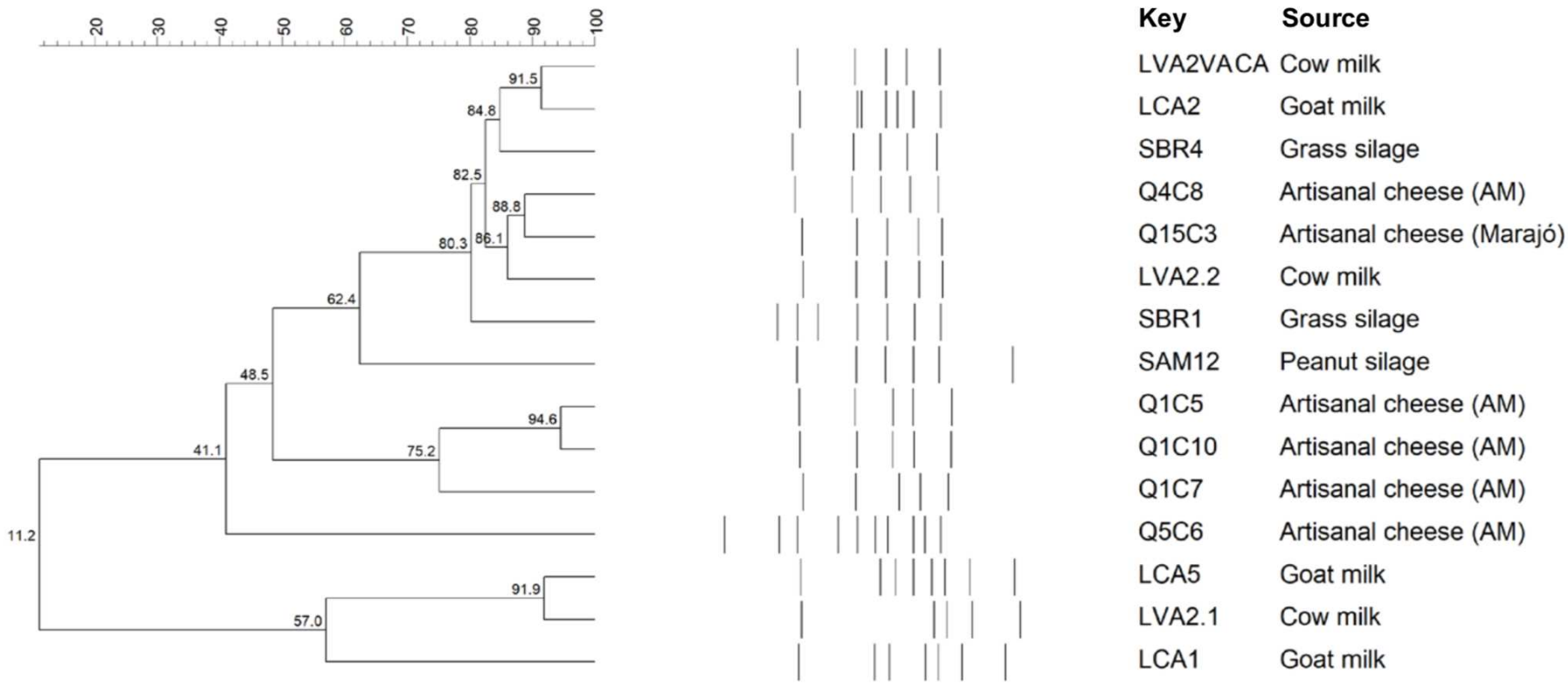

Figure 1. Dendrogram based on the UPGMA cluster in the REP-PCR fingerprints analysis for the 15 isolates of *L. lactis* subsp. *lactis* bv. diacetylactis.

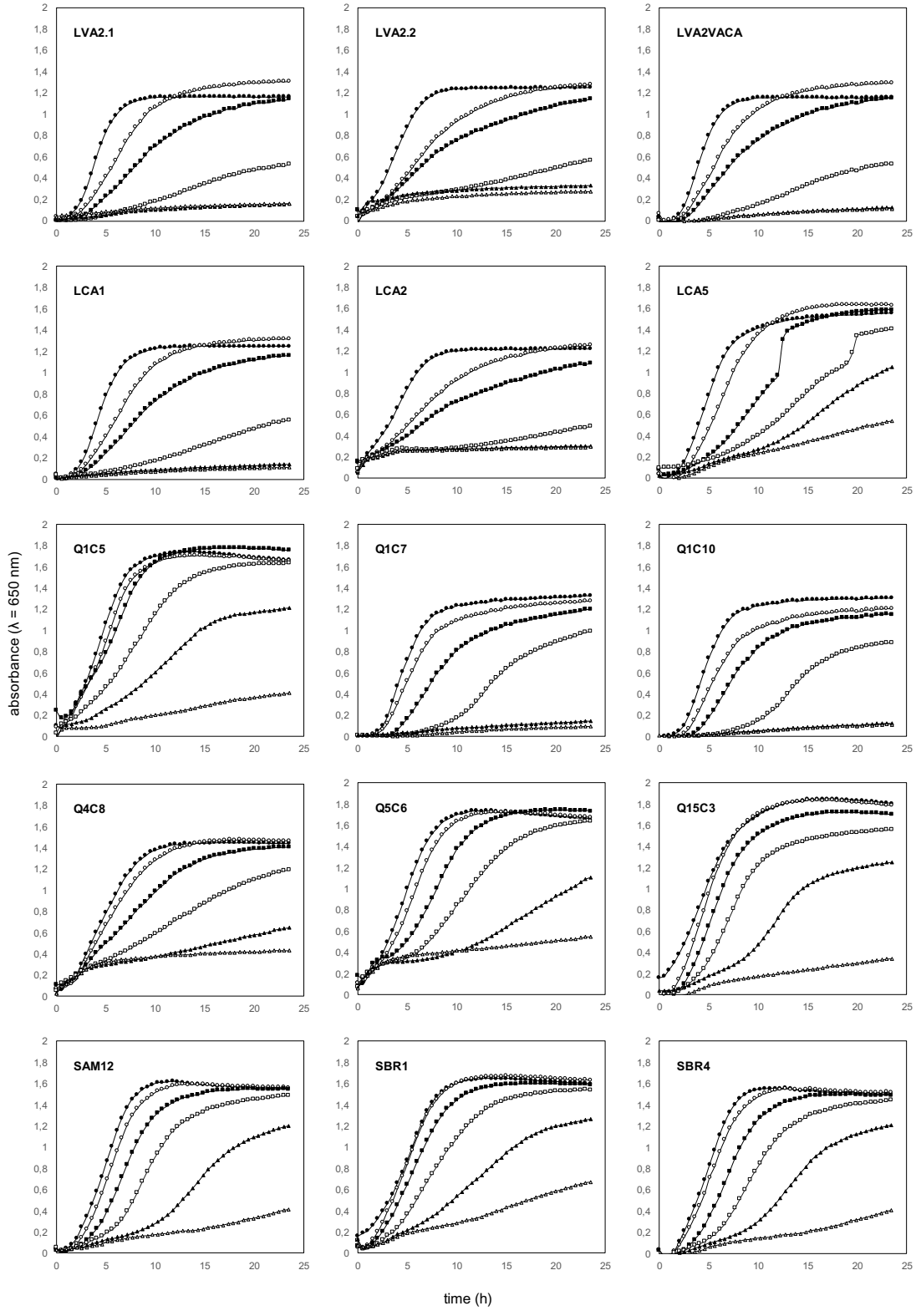

Figure 2. Growth curves of *L. lactis* subsp. *lactis* bv. diacetylactis isolates at different concentrations of NaCl. Growth in: ● - 0% NaCl; ○ - 2% NaCl; ■ - 4% NaCl; □ - 6% NaCl;▲ - 8% NaCl; Δ - 10% NaCl.

CAPÍTULO 2 – Ability of *Lactococcus lactis* subsp. *lactis* bv. diacetylactis strains to produce nisin

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Manuscript prepared for submission to *Probiotics and Antimicrobial Proteins* (IF 2.345)

Title page

Ability of *Lactococcus lactis* subsp. *lactis* bv. diacetylactis strains to produce nisin

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ABSTRACT

Lactococcus lactis subsp. *lactis* bv. diacetylactis has a major importance in dairy industry due to the production of aromatic compounds: diacetyl and acetoin. Bacteriocin production can be an additional beneficial feature presented by some strains. This study aimed to characterize the bacteriocinogenic potential of *L. lactis* subsp. *lactis* bv. diacetylactis strains obtained from dairy production systems. A panel of 15 *L. lactis* subsp. *lactis* bv. diacetylactis was subjected to PCR to detect bacteriocin-related genes (nisin, lacticins 481 and 3147, lactococcins A and 972), and further investigated by PCR and sequencing for the nisin operon. Cell free supernatants (CFS) of nisin-positive strains were tested by the spot-on-the lawn assay against a panel of 16 targets (*Listeria monocytogenes* - 4, *L. innocua* - 1, *Staphylococcus aureus* - 6, *Lactobacillus sakei* - 1, *L. lactis* - 4). Further, growth curves of 4 microbial targets (*L. monocytogenes* - 2, *S. aureus* - 2), alone and in the presence of the CFS of nisin producers, were obtained by optical density (λ = 650 nm). Eight strains presented positive results only for *nisA*, and only one strain (SBR4) presented the full nisin operon, confirmed by sequencing as similar to nisin Z. Only SBR4 presented inhibitory activity by the spot-on-the lawn assay against the 16 microbial targets. Growth curves of selected targets confirmed the inhibitory activity of the SBR4 strain, indicating its potential for nisin production. The study has demonstrated the inhibitory potential of a *L. lactis* subsp. *lactis* bv. diacetylactis strain, SBR4, due to its ability in produce nisin Z. Considering the technological properties of this strain, this inhibitory potential indicates an additional beneficial feature of SBR4 to be considered in the dairy industry.

Key-words: antimicrobial peptide; lactic acid bacteria; lantibiotic; nisin Z

INTRODUCTION

Lactococcus lactis subsp. *lactis* bv. diacetylactis is a lactic acid bacteria (LAB) of particular interest in the dairy industry due to its ability of fermenting citrate and producing diacetyl and acetoin, desirable flavoring compounds in specific ripened cheeses. This technological feature is very important for starter cultures, and strains possessing this characteristic can also be used in food preservation due to their potential in producing bacteriocins (Schleifer et al., 1985; Deegan et al., 2006). Some strains of *L. lactis* subsp. *lactis* bv. diacetylactis were already described as being able to produce lactococcin A (Stoddard et al., 1992), lactoccin D (Parente and Hill, 1992), licheniocin 50.2 (Cirkovic et al., 2016) and lactolisterin BU (Lozo et al., 2017). However, to date, only one strain has been identified as capable of producing nisin: *L. lactis* subsp. *lactis* bv. diacetylactis UL719, isolated from raw milk cheese (Ali et al., 1995; Meghrous et al., 1997).

Nisin presents a wide spectrum of antimicrobial activity, targeting foodborne pathogens like enterotoxigenic *Staphylococcus aureus* and *Listeria monocytogenes*, allowing its use as an effective method to extend the shelf life and assure the safety in different foods (Delves-Broughton, 1990; De Arauz et al., 2009; Bali et al., 2016). To date, only nisin (e.g. Nisaplin, Danisco) and pediocin ALTA 2431® (e.g. Microgard™, ALTA 2431, Quest Int.) have been commercialized as food preservatives (Simha et al., 2012; Silva et al., 2018). Accordingly, nisin has been applied in the food industry as a biopreservative for more than 50 years without inducing resistance in the potential microbial targets (Delves-Broughton, 1990; Deegan et al., 2006). Also, nisin was approved as a safe food preservative by Food and Agricultural Organization (FAO) and Food and Drug Administration (FDA), being licensed to be used in more than 60 countries (De Arauz et al., 2009; López-Cuellar et al., 2016).

Several variants of nisin have been described, with variation of amino acids and size of the molecule, and all of them present similar antimicrobial activity. Nisin A variant was the first to be described (Rogers, 1928; Gross and Morell, 1971), and it is the most widely studied one (Kuipers et al., 1995; Li and O'Sullivan, 2002; Lubelski et al., 2008; Piper et al., 2011; Perin et al., 2012; Perin et al., 2016; Mulkyte et al., 2017; Field et al., 2018). Other natural variants of nisin have been described: nisin Z (Mulders et al., 1991), nisin Q (Zendo et al., 2003), nisin U and U2 (Wirawan et al., 2006), nisin F (Kwaadsteniet et al., 2008), nisin P (Zhang et al., 2012; Wu et al., 2014), and nisin H (O'Connor et al., 2015). Nisin biosynthetic genes are located on a conjugative transposon (*Tn5276*) and they are typically organized in four operons: *nisABTCIPRK* (nisin structure and maturation), *nisI* (immunity), *nisRK* (regulation) and *nisFEG* (immunity) (Lubelski et al., 2008; Field et al., 2015). The strain *L. lactis* subsp. *lactis* bv. diacetylactis UL719 was characterized as being able to produce nisin Z (Ali et al., 1995; Meghrous et al., 1997).

Considering the technological relevance of *L. lactis* subsp. *lactis* bv. diacetylactis for the dairy industry, this study aimed to investigate the inhibitory potential of strains belonging to this biovar obtained from dairy production environment in Brazil, with special attention to their ability in producing nisin.

MATERIAL AND METHODS

Strains

A total of 15 *L. lactis* subsp. *lactis* bv. diacetylactis strains were included in this study. The strains were obtained from the bacteria culture collection at InovaLeite (Laboratory of Milk and Dairy Products, Universidade Federal de Viçosa), and they

were isolated from different ecosystems, including dairy production environment (grass and peanut silages, raw milk from cow and goat) and artisanal cheese production (manuscript under submission). All strains were previously identified as *L. lactis* subsp. *lactis* bv. diacetylactis by sequencing of a region from 16S rRNA, PCR assays targeting the identification of subspecies (*lactis*) and histidine biosynthesis operon, and phenotypic methods (fermenting citrate and producing diacetyl) (manuscript under submission).

Molecular characterization of bacteriocin production potential

The selected strains were cultured in de Man, Rogosa and Sharpe (MRS) broth (Oxoid Ltd., Basingstoke, England) at 30 °C for 18 h. Aliquots of 1 mL were centrifuged at 14,000 × *g* for 2 min, and the DNA of the cell pellets was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). PCR assays were conducted in order to identify the following bacteriocin related genes: nisin (Li and O'Sullivan, 2002), lacticin 481 (Rodríguez et al., 2000), lacticin 3147 (Perin et al., 2012), lactococcin 972 (Alegría et al., 2010) and lactococcin A (Rodríguez et al., 2000). PCR assays were performed as described by the authors and primer sequences, fragment sizes (bp) and positive controls are described in Table 1. The obtained PCR products were electrophoresed on 1.5% agarose gels (w/v), stained using Diamond dye solution (Promega) and visualized using a transilluminator LPIX (Loccus Biotecnologia, São Paulo, SP, Brazil).

Then, all strains were also subjected to PCR assays targeting genes enrolled in nisin production; primers sequences and target genes/regions for each assay are described in Table 2. *L. lactis* subsp. *lactis* DY13 (Lyofast DY13, Sacco, Cadorago, Italy) was used as a positive control for all reactions. Amplifications and electrophoresis of PCR

products were performed according to Olasupo et al. (1999), Li and O'Sullivan (2002), Ghrairi et al. (2004) and Veljovic et al. (2007).

Based on the obtained results, strains positive for *nisA* according Li and O'Sullivan (2002) were selected and subjected to sequencing in Macrogen Inc. (Seoul, South Korea). DNA sequences were analyzed and translated using the software SequencherTM 4.1.4 (Technology Drive, Ann Arbor, MI, USA) in order to identify similarities between the translated amino acid sequences and nisin A, Z, Q, U1, U2, P, F and H sequences previously deposited in GenBank (www.ncbi.nlm.nih.gov/Genbank/submit.html).

Phenotypic characterization of nisin production

Strains positive for *nisA* (Li and O'Sullivan, 2002) were cultured in MRS broth at 30 °C for 24 h and cell-free supernatant (CFS) was obtained by centrifugation at 10,000 × *g* for 10 min. The pH of the supernatant was adjusted to 6.5 with 1M NaOH and treated for 10 min at 80 °C. The CFS of nisin-positive strains were tested by the spot-on-the lawn assay against *L. monocytogenes* ATCC 7644, *L. monocytogenes* Scott A and *S. aureus* ATCC 6538 at 10^5 CFU/mL final concentration and inhibition zones larger than 2 mm diameter were considered as positive results (Cavicchioli et al., 2017). *L. lactis* subsp. *lactis* DY13 strain was used as a positive control.

Then, the CFS of the strains that presented antagonistic potential were used to evaluate the inhibitory spectrum by the spot-on-the lawn assay against a panel of 13 targets: *L. monocytogenes* ATCC 15313, ATCC 19112; *L. innocua* ATCC 33090; *S. aureus* ATCC 29923, ATCC 700698, ATCC 43300, ATCC 14458, ATCC 12598; *Lactobacillus sakei* ATCC 15521; *L. lactis* subsp. *lactis* ATCC 13675, ATCC 19435; *L. lactis* subsp. *cremoris* ATCC 19257, CNRZ 481.

Further, four microbial targets (*L. monocytogenes* ATCC 7644, ATCC 15513; *S. aureus* ATCC 14458, ATCC 12598) were cultured in brain heart infusion (Oxoid) at 35 °C for 24 and 100 µL were transferred to 96-well microtiter plates at approximate concentration of 10^5 CFU/mL. Then, CFS aliquots of the selected nisin producer strain were added to each well, and the plates were incubated at 35 °C for 24h in MultiskanTM GO Microplate Spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA). The optical densities (OD) of the cultures were measured in each 15 min (λ = 650 nm) (Turcotte et al., 2004). This assay was conducted in three replicates and wells without the CFS were considered as controls.

RESULTS AND DISCUSSION

LAB are found in different ecological niches, and as a result of their efforts to adapt and survive, they produce various secondary metabolites, among which are bacteriocins (Lozo et al., 2017). PCR assays were undertaken using specific primers for genes of the most common lactococcal bacteriocins, and amplicons of the expected size for lacticin 481, lacticin 3147, lactococcin 972, and lactococcin A were not obtained (data not shown). In contrast, 8 of the 15 strains of *L. lactis* subsp. *lactis* bv. diacetylactis produced an amplicon of the expected size for nisin. Alegría et al. (2010) described bacteriocins genes in *L. lactis* from traditional Spanish cheeses made of raw milk, 11 of the 17 strains were positive for nisin and five for lactococcin 972, though no positive strains were observed for lacticin 3147, lacticin 481, and lactococcins A. Nisin biosynthetic genes are located on a conjugative transposon (*Tn5276*) and they are typically organized in four operons: *nisABTCIPRK*, *nisI*, *nisRK* and *nisFEG* (Lubelski et al., 2008). The total operon of nisin consists in 11 genes encoding by

proteins and we investigated that the operon is present in *L. lactis* subsp. *lactis* bv. diacetylactis strain by amplifying each of the genes enrolled in nisin production. Table 3 presents the PCR results for *Tn5276* genes and regions in the 15 bv. diacetylactis strains included in this study. For the pre-peptide gene *nisA*, 8 strains presented positive results, based on three different primers sets (Table 3). *nisA* gene encodes the nisin precursor protein (NisA) and positive result for the presence of this gene in these strains is not directly indicative of nisin production, because *Tn5276* must include all genes of nisin operon (Lubelski et al., 2008).

Following ribosomal synthesis by the precursor peptide (pre-NisA), prenisin is post-translational modified via the *nisBC* modification machinery (Alkhatib et al., 2012), essential for the maturation and biosynthesis of nisin (Khusainov et al., 2011). NisB is an enzyme that dehydrates serines and threonines residues in the prepeptide (Sen et al., 1999), and NisC, a zinc-dependent metalloprotein that cyclizes dehydrated residues to C-terminally located cysteine residues to form methyl-lanthionine or lanthionine rings (Koponen et al., 2002; Alkhatib et al., 2012). In order for these biosynthetic modifications to take place, the fully modified prenisin, but inactive, called mature NisA (mNisA), is exported out of the cell membrane by NisT, an ABC transporter that forms a membrane associated complex with *nisB* and *nisC* (Qiao and Saris, 1996). After exporting, mNisA is activated proteolytically by cleavage of the leader peptide and this process is performed by the cell-membrane anchored protease, NisP (Kuipers et al., 1993; Lagedroste et al., 2017).

Only SBR4 strain presented positive results for genes related to maturation and biosynthesis (*nisB* and *nisC*) and transport (*nisT*) (Table 3). For processing gene (*nisP*), 8 strains presented positive results, the same that were positive for *nisA* (Table 3).

Thus, only the SBR4 strain was characterized as possessing the ability of producing extracellular mature and active nisin peptide.

The nisin biosynthesis is auto-regulated by the two-component regulatory sensor histidine kinase system NisRK, which allows self-induction of the *nisA* promoter by the mature nisin product (Chatterjee et al., 2005; Cheigh and Pyun, 2005). In this study, nine strains presented positive results for *nisRK* (Table 3), demonstrating the presence of this regulatory mechanism. It is interesting that Q4C8 strain was not positive for *nisA* or other genes and present positive results for *nisRK* and *nisF* (Table 3).

Nisin operon contains four genes that encode self-immunity proteins, acting in a cooperative system: *nisF, nisE,* and *nisG* encodes an ABC transporter with an immunity function against nisin (Alkhatib et al., 2014), and *nisI*, which encodes for NisI lipoprotein that also contributes to the protection against nisin (Siegers and Entian, 1995). In this research, we use specific primers for *nisF* that is part of the *nisFEG* operon (Table 2), and eight strains present positive results (Table 3). For *nisI*, only SBR4 strain was positive (Table 3). The total immunity is achieved only when *nisI* and *nisFEG* are expressed (Stein et al., 2003), but the mechanisms by which both participate in the protection against nisin are still unknown. According Ra et al. (1999), *nisI* and *nisFEG* are very important to provide immunity.

Of all 15 lactococcal strains tested, *L. lactis* subsp. *lactis* bv. diacetylactis SBR4 showed viability in the complete expression of nisin genes, indicating the integrity of *Tn5276*. The positive control *L. lactis* subsp. *lactis* DY13 also presented the integrity. The absence of any of the genes enrolled in this operon can sabotage nisin biosynthesis (Li and O'Sullivan, 2002). In this study, we demonstrated that the nisin gene cluster is present in this strain by amplifying each of the genes enrolled in nisin production (*nisABTCIPRK*) and also *nisF*, which is part of the downstream *nisFEG* operon

involved in nisin immunity. The data indicated that the nisin gene cluster is most likely fully intact in *L. lactis* subsp. *lactis* bv. diacetylactis SBR4 strain when compared to the other strains.

To verify the integrity of the structural gene for nisin, PCR products for nisA were sequenced and the translated amino acid sequences are presented in Figure 1. The amino acid sequences were compared with nisin A, Z, Q, U1, U2, P, F and H, already reported in GenBank, and with nisin variant sequences previously characterized from two wild strains of *L. lactis* subsp. *lactis* also isolated from raw goat milk by Perin and Nero (2014).

Prenisin contains 57 amino acid residues, which after translation are targeted to a modification machinery. The N-terminal leader peptide corresponding the first 23 amino acid residues that are crucial in recognition of unmodified prenisin by the modification and transport proteins (Kuipers et al., 1993; Siegers et al., 1996; Kuipers et al., 2006), and the other 34 amino acids encode the active nisin (Alkhatib et al., 2012). Considering the leader peptide (amino acids -23 to -1, Fig. 1), one variation was identified at position -7 in six translated sequences of nisin from the *L. lactis* subsp. *lactis* bv. diacetylactis strains, except Q5C6 and SBR4, an aspartic acid (negative-charged amino acid) was replaced by an asparagine (uncharged amino acid). The same variation was found in nisin U1 and nisin H and in the two strains of *L. lactis* subsp. *lactis* (GLc03; GLc04) (Figure 1). According Khusainov et al. (2013), leader substitutions in the region SKKD (amino acid -10 to -7) do not interfere with the activity of the peptide. The leader peptide is a recognition signal for the modification enzymes NisB and NisC (Khusainov et al., 2013; Khusainov et al., 2015) and the transporter NisT (Meers et al., 1994). Besides that, this leader peptide keeps the fully modified precursor nisin inactive (Kuipers et al., 1993; Meers et al., 1994). NisB,

NisC, and NisT can also modify and transport peptides unrelated to nisin provided that the nisin leader peptide is present at the N-terminus (Kuipers et al., 2004; Rink et al., 2007; Khusainov et al., 2015). The amino acid sequence of the mature peptide (amino acids +1 to +34) from seven strains presented two variations (Figure 1) when compared to nisin A (the first nisin variant described): a leucine was replaced by a valine at position +16 and a histidine was replaced by an asparagine at position +27. Similar to other lantibiotics, nisin contains several unusual amino acids as a result of enzymatic post-translational modifications (Smith and Hillman, 2008). Anyway, six strains were identified as capable of producing a novel nisin variant because their amino acid sequences were diverse at the -7, +16 and +27 positions of the other nisin variants already described (Figure 1). Perin and Nero (2014) described the same amino acid sequence in *L. lactis* subsp. *lactis* GLc03 strain. In this study, the strain Q5C6 also had potential to produce a new variant of nisin, with variation in position +16 and +27.

However, the SBR4 strain presented only the variation at position +27 (Figure 1): an uncharged amino acid (asparagine) was replaced by a positive electrically charged and basic amino acid (histidine), responsible for increasing its inhibitory spectrum due to its improved diffusion capacity in culture media (Mulders et al., 1991). The predicted mature peptide sequence encoded by SBR4 strain was found to be very similar to other NisA proteins, with same positions for serine, threonine and cysteine residues. This indicates that the bacteriocin produced by strain SBR4 is a natural variant of nisin, called nisin Z. Nisin Z, the closest variant of nisin A, was firstly described as being produced by *L. lactis* NIZO 22186 (Mulders et al., 1991; De Vos et al., 1993). Nisin A and Z share similar properties as antimicrobials, but nisin Z has a higher rate of diffusion and solubility under neutral pH conditions (De Vos et al., 1993; Laridi et al.,

2003). The amino acid sequence of the positive control, *L. lactis* subsp. *lactis* DY13, indicating that it produces nisin A (Figure 1).

Although the occurrence of nisin Z in many strains of *L. lactis* has been reported from different origins (Olasupo et al., 1999; Ghrairi et al., 2004; Biscola et al., 2013; Dal Bello et al., 2012; Perin et al., 2012; Zhang et al., 2014), this is the first time that a nisin Z producing bv. diacetylactis strain has been detected in grass silage. A nisin Z producer, *L. lactis* subsp. *lactis* bv. diacetylactis UL719, isolated from raw milk cheese is described in the literature (Ali et al., 1995; Meghrous et al., 1997). Considering the technological relevance of *L. lactis* subsp. *lactis* bv. diacetylactis for the dairy industry due to the production of aromatic compounds, the additional beneficial characteristic of producing nisin Z must be considered as a significant feature identified in the SBR4 strain, highlighting its potential as be explored as starter and biopreservative strain.

For phenotypic characterization of nisin production, the CFS of *nisA* positive strains were tested by spot-on-the lawn assay against *L. monocytogenes* ATCC 7644, *L. monocytogenes* Scott A and *S. aureus* ATCC 6538, and only SBR4 strain present inhibition zones (data not shown). SBR4 also presented full integrity of *Tn5276*, and the amino acid sequence confirm its ability in producing nisin Z variant. Strains that did not present *Tn5276* integrity did not present inhibition to the target strains. Subsequently, SBR4 CFS was used to evaluate the inhibitory spectrum, and all 13 microbial targets were inhibited. Growth curves of selected microbial targets confirmed the inhibitory activity of the SBR4 strain, indicating its potential for nisin production (Figure 2). During the lag phase following inoculation of bacteria into fresh medium, many physiological functions must be restored, and an adaptation process must therefore take place (Derzelle et al., 2003). The obtained data indicated a bacteriostatic effect of the CFS produced by SBR4, what can be considered as an

important adaptation role for the bacterocin producer. The inhibitory actitiy of SBR4 CFS were higher when compared to the inhibitory activity of the CFS produced by the control DY13, considering all microbial targets included in the study (Figure 2), demonstrating its inhibitory potential compared to a commercial nisin-producing culture used in industry (Figure 2). SBR4 was characterized as being able to produce nizin Z (Figure 1), a bacteriocin that present a high diffusion rate and solubility at neutral pH conditions (De Vos et al., 1993; Laridi et al., 2003); these characteristics were observed in the agar spot assay, once the CFS produced by SBR4 presented larger inhibition halos that the CFS produced by DY13, a know nisin A producer.

Considering both approaches to inhibitory characterization of SBR4, microplate inhibition assay can be considered more sensitive than the agar spot assay. Assays using 96-well microplate systems have been proposed to quantify bacteriocin activity in CFS, while the precision of results obtained by the agar diffusion assay may be jeopardized when the inhibition zone is unclear or not perfectly circular (Turcotte et al., 2004). According to the results obtained by the microplate assay, presented higher inhibitory activity against *S. aureus* when compared to *L. monocytogens* (Figure 2). *S. aureus* is a pathogen frequently associated with cases and outbreaks of food poisoning due to its ability to produce enterotoxin (Viçosa, 2018) and studies have revealed its presence and/or survival in dairy products (Alegria et al., 2009; Jamali et al., 2015; Basanini et al., 2017). However, research have demonstrated the positive effect of nisin against *S. aureus* (Rilla et al., 2004; Pinto et al., 2011; Felicio et al., 2015; Perin et al., 2015; Wang et al., 2016), as observed in the present study.

CONCLUSIONS

This work provides insight into a new strain, *L. lactis* subsp. *lactis* bv. diacetylactis SBR4, to produce nisin Z. Its technologic features, genetic and spectrum of activity characteristics point as a good candidate to be a safe and natural food preservative to be considered in the dairy industry.

ACKNOWLEDGMENTS

CNPq, CAPES (financial code 001) and FAPEMIG.

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Table 1. Information of the PCR protocols for identification of bacteriocins genes in *L. lactis* subsp. *lactis* bv. diacetylactis strains.

Gene/bacteriocin	Primers sequences	Fragment size (bp)	Positive controls	References
Nisin	F: GGATAGTATCCATGTCTG R: CAATGATTTCGTTCGAAG	300	*L. lactis* subsp. *lactis* DY13	Li and O'Sullivan (2002)
Lacticin 481	F: TCTGCACTCACTTCATTAGTTA R: AAGGTAATTACACCTCTTTTAT	360	*L. lactis* subsp. *cremoris* CNRZ481/45	Rodríguez et al. (2000)
Lacticin 3147	F: AAATTAATGAGACAGACTTTG R: CATCATCCATAACTATATTTG	105	*L. lactis* subsp. *lactis* DPC3147	Perin et al. (2012)
Lactococcin 972	F: TTGTAGCTCCTGCAGAAGGAACATGG R: GCCTTAGCTTTGAATTCTTACCAAAAG	350	-	Alegría et al. (2010)
Lactococcin A	F: CAATCAGTAGAGTTATTAACATTTG R: GATTTAAAAAGACATTCGATAATTAT	771	-	Rodríguez et al. (2000)

Table 2. Primers used to detect nisin related genes and regions from the total DNA obtained from *L. lactis* subsp. *lactis* bv. diacetylactis strains.

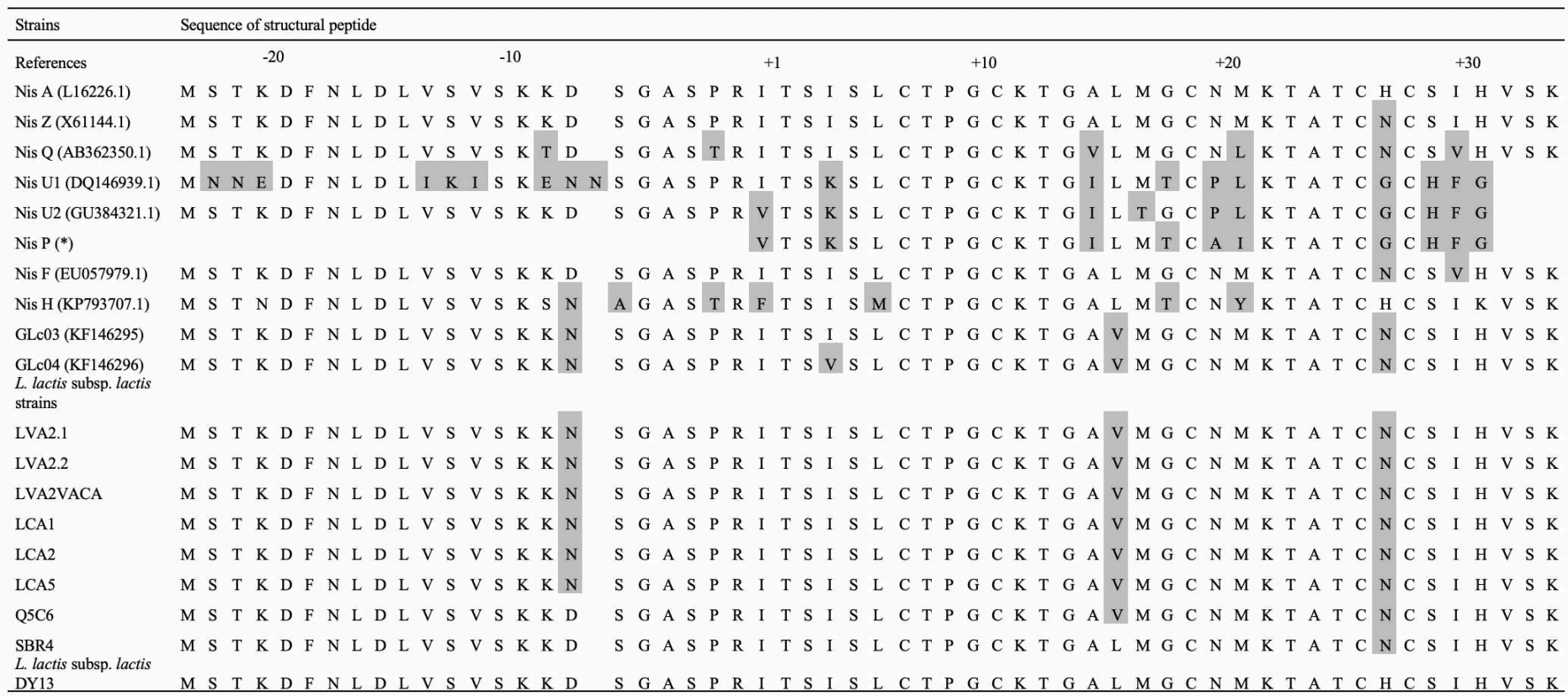
Primer set	Primers sequences	Target	Function [a]	Fragment size (bp)	References
P01	F: GGATAGTATCCATGTCTG	*nisA*	expression	300	Li and O'Sullivan (2002)
	R: CAATGATTTCGTTCGAAG				
P02	F: CCTCGACGATACCATCAC	*nisA*	expression	745	Veljovic et al. (2007)
	R: CTCCGTTTATCGTTTGGAG				
P03	F: CGGCTCTGATTAAATTCTGAAG	*nisA*	expression	399	Olasupo et al. (1999)
	R: CGGTTGAGCTTTAAATGAAC				
P04	F: CGCTTTGCTATGGAGACGAAT	*nisB*	maturation	480	Li and O'Sullivan (2002)
	R: GAGCTCCTATGCCAAATGTA				
P05	F: AGAGAAGTTATTTACGATCAAC	*nisB*	maturation	457	Olasupo et al. (1999)
	R: ATCTGACAACAAATCTTTTTGT				
P06	F: GAAGAATACATGAAATGAGG	*nisT*	transport	285	Li and O'Sullivan (2002)
	R: TAACTTTCCAGCTGTCCC				
P07	F: TTCAGAGCAATATGAGG	*nisC*	maturation	1289	Olasupo et al. (1999)
	R: TATTAAGGCCACAATAAG				
P08	F: ATTGTGGCCTTAATAGGG	*nisI*	immunity	264	Li and O'Sullivan (2002)
	R: TAGCGACTTGTCAGAAGC				
P09	F: GGATTTGGTATCTGTTTCGAAG	*nisP*	processing	600	Ghrairi (2004)
	R: TCTTTCCCATTAACTTGTACTGTG				
P10	F: CAGTGCCATGGGTAAAAAATATTCAATGCG	*nisRK*	regulation	523	Veljovic et al. (2007)
	R: CTTAGAGAATTCTCTAATGAG				
P11	F: CAGGTGCTACAAGATATCAG	*nisF*	immunity	441	Li and O'Sullivan (2002)
	R: ACAACTCCGCAATACCATCAG				

[a] According Alkhabit (2012).

Table 3. PCR results for nisin related genes in *L. lactis* subsp. *lactis* bv. diacetylactis strains.

Strain	Origin	Primer set and target[a]										
		P01 *nisA*	P02 *nisA*	P03 *nisA*	P04 *nisB*	P05 *nisB*	P06 *nisT*	P07 *nisC*	P08 *nisI*	P09 *nisP*	P10 *nisRK*	P11 *nisF*
LVA2.1	cow milk	+	+	+	-	-	-	-	-	+	+	+
LVA2.2	cow milk	+	+	+	-	-	-	-	-	+	+	+
LVA2VACA	cow milk	+	+	+	-	-	-	-	-	+	+	+
LCA1	goat milk	+	+	+	-	-	-	-	-	+	+	+
LCA2	goat milk	+	+	+	-	-	-	-	-	+	+	+
LCA5	goat milk	+	+	+	-	-	-	-	-	+	+	+
Q1C5	artisanal cheese (AM)[b]	-	-	-	-	-	-	-	-	-	-	-
Q1C7	artisanal cheese (AM)	-	-	-	-	-	-	-	-	-	-	-
Q1C10	artisanal cheese (AM)	-	-	-	-	-	-	-	-	-	-	-
Q4C8	artisanal cheese (AM)	-	-	-	-	-	-	-	-	-	+	+
Q5C6	artisanal cheese (AM)	+	+	+	-	-	-	-	-	+	+	-
Q15C3	artisanal cheese (Marajó)[c]	-	-	-	-	-	-	-	-	-	-	-
SAM12	peanut silage	-	-	-	-	-	-	-	-	-	-	-
SBR1	grass silage	-	-	-	-	-	-	-	-	-	-	-
SBR4	grass silage	+	+	+	+	+	+	+	+	+	+	+
DY13[d]	-	+	+	+	+	+	+	+	+	+	+	+

[a] Primers sequences and target described in Table 2; [b] artisanal cheese obtained from the Amazonian region, Brazil; [c] artisanal cheese obtained from the Marajó Island, Northern region of Brazil; [d] Positive control *L. lactis* subsp. *lactis* DY13; + = positive; - = negative.



Strains	Sequence of structural peptide
References	-20 -10 +1 +10 +20 +30
Nis 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
Nis 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
Nis 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
Nis 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
Nis 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
Nis P (*)	V T S K S L C T P G C K T G I L M T C A I K T A T C G C H F G
Nis 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
Nis H (KP793707.1)	M S T N D F N L D L V S V S K S N A G A S T R F T S I S M C T P G C K T G A L M T C N Y K T A T C H C S I K V S K
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
L. lactis subsp. *lactis* strains	
LVA2.1	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
LVA2.2	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
LVA2VACA	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
LCA1	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
LCA2	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
LCA5	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
Q5C6	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 V M G C N M K T A T C N C S I H V S K
SBR4	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
L. lactis subsp. *lactis* DY13	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

Figure 1. Amino acid sequences deduced from the nisin gene sequencing of eight *L. lactis* subsp. *lactis* bv. diacetylactis strains.

Compared to the sequences of nisin A, Z, Q, U1, U2, P, F and H and two strains of *L. lactis* subsp. *lactis* (GLc03; GLc04) isolated from raw goat milk by Perin and Nero (2014). The leader peptide is composed by 23 amino acids, followed by amino acids present in the mature peptide. Amino acids highlighted in grey indicate variations when compared to the nisin A reference. The complete amino acid sequences from the eight wild strains have been deposited in GenBank (accession numbers: MK470548-MK470555). *Described by Zhang et al. (2012) and Wu et al. (2014).

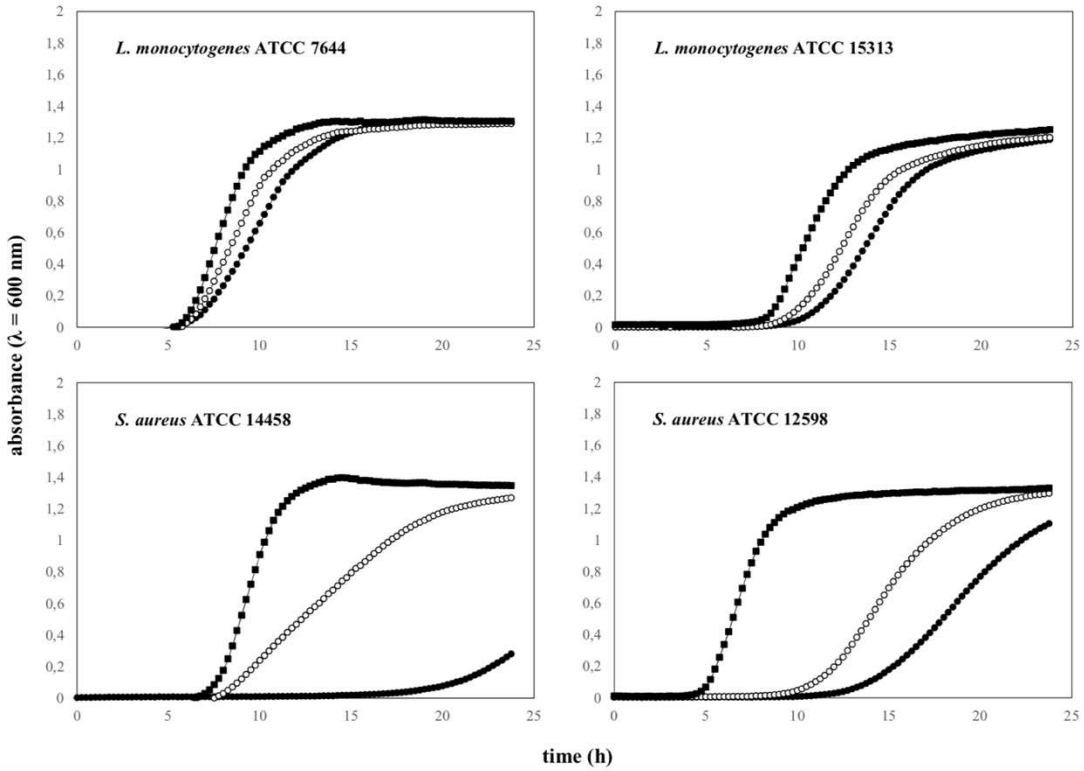


Figure 2. Growth curves of 4 microbial reference targets alone and in the presence of the CFS of nisin producers.

Growth in: ● - microbial reference target in the presence of the CFS of *L. lactis* subsp. *lactis* bv. diacetylactis SBR4 strain; ○ microbial reference target in the presence of the CFS of *L. lactis* subsp. *lactis* DY13 strain; ■ - microbial reference target alone.

CONCLUSÕES GERAIS E PERSPECTIVAS

- Dos 23 isolados de *L. lactis* subsp. *lactis*, 15 foram identificados como pertencentes ao biovar diacetylactis.
- Os 15 isolados de *L. lactis* subsp. *lactis* bv. diacetylactis demonstraram potencial tecnológico favorável e possível uso em culturas starter na indústria de laticínios, mas a aplicação tecnológica destas depende das funções que os micro-organismos irão exercer sobre o produto final, bem como sobre o produto ao qual ele será adicionado.
- Os isolados de *L. lactis* subsp. *lactis* bv. diacetylactis apresentaram ausência de genes envolvidos na produção de lacticina 481, lacticina 3147, lactococcina 972 e lactococcina A, e apresentaram presença de genes envolvidos na produção de nisina.
- Na identificação dos genes do operon de nisina, a cepa *L. lactis* subsp. *lactis* bv. diacetylactis SBR4 apresentou a integridade do transposon conjuntivo *Tn5276.*
- O sequenciamento de amino ácidos demonstrou que a variante de nisina produzida pela cepa SBR4 corresponde a nisina Z, as demais cepas apresentaram variações nas posições -7, +16 e +27.
- *L. lactis* subsp. *lactis* bv. diacetylactis SBR4 demonstrou amplo espectro inibitório, principalmente frente a *S. aureus*, indicando seu potencial de utilização como bioconservante.
- Considerando-se o potencial biotecnológico e a habilidade em produzir nisina da cepa *L. lactis* subsp. *lactis* bv. diacetylactis SBR4, esta pode ser usada para

constituir uma cultura starter ou mista, cujas características de produção de queijos podem ser avaliadas no futuro.

- A nisina Z produzida por *L. lactis* subsp. *lactis* bv. diacetylactis SBR4 requer análises para melhorar a performance da sua produção por meio da padronização das condições empregadas.

- A cepa *L. lactis* subsp. *lactis* bv. diacetylactis SBR4 deve receber atenção especial, com necessidade de análises que assegurem sua utilização em alimentos, para então tornar-se uma cultura starter considerada de suma importância como ferramenta bioconservadora e biotecnológica.