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BIOPROSPECTION OF TECHNOLOGICAL, PROBIOTIC POTENTIAL AND SAFETY ASSESSMENT OF ENDOGENOUS LACTIC ACID BACTERIA ISOLATED FROM BRAZILIAN ARTISANAL CHEESES

BIOPROSPECÇÃO DO POTENCIAL TECNOLÓGICO, PROBIÓTICO E AVALIAÇÃO DA SEGURANÇA DE BACTÉRIAS LÁTICAS ENDÓGENAS DE QUEIJOS ARTESANAIS BRASILEIROS

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Bioprospecção do potencial tecnológico, probiótico e avaliação da segurança de bactérias láticas endógenas de queijos artesanais brasileiros

Thesis presented to the Faculty of Food Engineering of the University of Campinas in a partial fulfilment of the requirements for the degree of doctor in Food Science.

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Orientador: Prof. Dr. ANDERSON DE SOUZA SANT'ANA

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RESUMO

Os queijos artesanais brasileiros (QAB) apresentam características singulares que podem refletir a história, a cultura e o estilo de vida das comunidades tradicionais produtoras. A microbiota endógena destes queijos, constituída principalmente por bactérias láticas (BAL), atua nas reações bioquímicas precursoras de texturas, aromas e sabores distintos. O objetivo deste estudo foi estudar as propriedades tecnológicas e probióticas de BAL isoladas a partir de QAB produzidos nas 5 regiões do país. No primeiro capítulo são reportados resultados da avaliação do potencial tecnológico (taxa de acidificação, proteólise, lipólise, atividade antimicrobiana e crescimento em diferentes condições de pH, sais biliares e cloreto de sódio) de 1.002 cepas de BAL isoladas a partir de 578 amostras de QAB. Para isso foi realizado um *screening* de alta performance em microplacas de 96 poços e os dados analisados no software R. O intuito foi selecionar as melhores cepas (n=220) com potencial para aplicação em culturas lácteas. No segundo capítulo, são apresentados resultados sobre a ocorrência de cepas do gênero *Enterococcus* e aspectos relacionados à sua segurança e potencial aplicação como culturas lácteas, devido ao papel controverso deste gênero em alimentos. No terceiro capítulo, são apresentados resultados de testes relacionados à formação de aroma, textura e produção de antimicrobianos de origem proteica das 220 BAL selecionadas. No quarto capítulo são reportados os resultados dos testes de potencial probiótico das 220 BAL, avaliado por meio da resistência à condições encontradas no trato gastrointestinal (TGI), propriedades de adesão e presença de genes de virulência. Uma cepa de *Lactobacillus plantarum* (1QB77) foi escolhida e empregada na produção de microqueijos em co-cultura com coquetéis de cepas de *Listeria monocytogenes* e *Staphylococcus aureus*. O quinto capítulo reporta resultados sobre a aplicação do método de engenharia evolutiva (EV) para a cepa 1QB77 a fim de melhorar sua performance na etapa inicial de acidificação do leite. Os resultados mostraram uma grande diversidade de BAL com potencial tecnológico, pertencentes aos gêneros *Lactobacillus*, *Lactococcus*, *Leuconostoc* e *Pediococcus*. A espécie de *L plantarum* foi a mais recorrente nos QAB, demonstrando grande heterogeneidade intra-espécie, de acordo com a região de isolamento. Houve associação entre algumas propriedades das BAL e a fonte de isolamento, como a produção de antimicrobianos de origem proteica ativos contra *L. monocytogenes* e os queijos Minas Artesanal e a produção de enzimas lipolíticas com os queijos do Marajó. A associação de métodos de *screening* de alto rendimento e análise multivariada proporcionou a seleção racional de um elevado número de cepas, agrupadas de acordo com seu potencial probiotico, biopreservativo ou como culturas adjuntas para potencial aplicação na produção de queijos, podendo contribuir para a segurança, qualidade e valorização de culturas regionais. O gênero *Enterococcus* foi considerado componente natural da microbiota de QAB, principalmente dos queijos Marajó, Coalho e Manteiga e 63% apresentaram potencial para aplicação como culturas iniciadoras e/ou biopreservadoras, devido a não produção de hemolisinas e sensibilidade a vancomicina. A cepa *Lb. plantarum* 1QB77 preencheu os principais critérios *in vitro* para avaliação de potencial probiótico, reduzindo em até 4 unidades logarítmicas a contagem dos patógenos testados ao longo da maturação dos microqueijos e após submissão às condições simuladas do TGI. O método de EV empregado para melhorar a performance da cepa *Lb. plantarum* 1QB77 aumentou em seis vezes a taxa de acidificação do leite das cepas evoluídas, em comparação com as parentais (não evoluídas), e aumentou em 90% a viabilidade desta cepa quando exposta à condições simuladas do TGI. O protocolo de produção de microqueijos (~ 190 mg) desenvolvido neste estudo apresentou resultados dentro dos apresentados na produção em larga escala (0,5-1 kg) para queijos de média umidade, configurando excelente alternativa para estudos envolvendo novas formulações, novas culturas, porém, com redução drática dos custos. Em suma, os QAB demonstraram ser excelentes fontes de bactérias láticas endógenas com potencial para serem aplicadas como culturas adjuntas ou biopreservativas na produção de produtos lácteos.

Palavras-chave: Queijos brasileiros, potencial tecnológico, probiótico, segurança de alimentos, qualidade de alimentos, engenharia evolutiva, *Enterococci*.

ABSTRACT

Brazilian artisanal cheeses (BAC) have unique characteristics, which reflect the history, culture, and lifestyle of traditional producing communities. The endogenous microbiota of these cheeses is mainly composed of lactic acid bacteria (LAB). These microorganisms play a crucial role in biochemical reactions that precede different textures, aromas, and flavors. This study aimed to investigate the technological and probiotic properties of LAB isolated from BAC produced in the five regions of Brazil. Chapter one shows results about the technological potential (acidification rate, proteolysis, lipolysis, antimicrobial activity, and growth under different pH conditions, bile salts, and sodium chloride) of 1,002 BAL strains isolated from 578 samples of BAC. For this purpose, high-throughput screening was performed using 96-well microplates, and all data analyzed using software R. The best strains (n=220) were selected due to its potential to be applied in dairy cultures. Chapter two aimed to investigate the occurrence of *Enterococcus* sp. strains and to access its safety and potential application as dairy cultures, due to the controversial role of *Enterococci* sp. in food. In chapter three, properties related to diacetyl (related to flavor), exopolysaccharide (EPS) (related to texture), and antimicrobials (with protein origin) formation of selected LAB (n =220) was accessed. Chapter four focused on studying some probiotic properties of the same LAB strains (n=220), as resistance to conditions found in the gastrointestinal tract (GIT), analysis of adhesion, and presence of virulence genes. Besides, a strain of *Lactobacillus plantarum* (1QB77) was chosen and used as adjunct culture in the manufacturing of microcheeses, in co-culture with cocktails containing *Listeria monocytogenes* and enterotoxigenic *Staphylococcus aureus* strains, separately. The fifth chapter aimed to apply a method called evolutionary engineering (EV) (or adaptative engineering) in order to improve the performance of *Lb. plantarum* (1QB77) in the initial step of the cheese process (acidification of milk). The results showed a great diversity of LAB with technological potential belonging to *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Pediococcus* genus. The *Lb. plantarum* species was the most recurrent in BAC, showing a great heterogeneity intra-species, according to cheese and region of isolation. There was an association between some properties of LAB and source of isolation, such as the production of antimicrobials with protein origin active against *L. monocytogenes* and Minas Artesanal cheeses and production of lipolytic enzymes with cheeses from Marajó.

The association between high-performance screening methods and multivariate analysis provided a rational selection of a high number of strains, making it possible to group them according to their features in probiotic, biopreservative, and adjunct cultures, which may contribute to safety, quality and enhancement of regional cultures in the dairy process. The *Enterococcus* genus was considered a natural component of BAC microbiota, mainly in Marajó, Coalho, and Manteiga cheeses, and 63% had the potential for application as a starter or biopreservative cultures, due to the absence of hemolysins and susceptibility to vancomycin. Strain *Lb. plantarum* 1QB77 met the main in vitro criteria for the evaluation of probiotic potential being able to reduce counts of pathogens tested along microcheeses process by up to 4 logarithmic units from the whole process (22 days of ripening) after submission to the simulated conditions of the GIT. The EV method used to improve the performance of *Lb. plantarum* 1QB77 increased milk acidification rates of evolved strains in six times, compared to parental (non-evolved) strains, and increased the viability of evolved strains by 90% when exposed to simulated GIT conditions. The microcheese production protocol (~ 190 mg) developed in this study showed results within those presented in large scale production (~ 0.5-1 kg) for medium moisture cheeses, configuring an excellent alternative for studies involving new formulations, new cultures, but with a drastic reduction in costs. In short, BAC has proved to be an excellent source of endogenous lactic acid bacteria with high potential to be applied as an adjunct or biopreservative cultures in the production of dairy products.

Keywords: Brazilian artisanal cheeses, technological potential, probiotics, food safety, food quality, evolutionary engineering, *Enterococci.*

SUMÁRIO

INTRODUÇÃO GERAL

Segundo o Ministério da Agricultura, Pecuária e Abastecimento, define-se queijo como “o produto fresco ou maturado que se obtém por separação parcial do soro do leite, ou leite reconstituído ou de soros lácteos, coagulados pela ação física do coalho, de enzimas específicas, de bactéria específica, de ácidos orgânicos, isolados ou combinados, todos de qualidade apta para uso alimentar, com ou sem agregação de substâncias alimentícias e/ou especiarias e/ou condimentos, aditivos especificamente indicados, substâncias aromatizantes e matérias corantes” (BRASIL, 1996).

Estima-se que mais de 1000 tipos de queijo são produzidos pelo mundo, os quais apresentam diferentes formas, texturas e aromas. Tais diferenças são influenciadas pelo tipo de matéria-prima utilizada (leite de vaca, búfala, ovelha), raça do animal, solo, clima, microclima e tecnologia de fabricação do queijo empregada. A técnica de fabricação de queijos varia de produtor para produtor, mas os princípios básicos mantiveram-se inalterados por milhares de séculos. As seguintes etapas são normalmente empregadas no processamento de queijos: coleta e seleção do leite, que pode ser submetido ou não ao processo de pasteurização para a eliminação de patógenos; coagulação do leite pela adição de culturas láticas ou enzimas coagulantes (renina); separação entre o coágulo e o soro; modelagem e salga; estocagem e maturação do queijo, etapas essenciais para a diferenciação das características organolépticas e de textura entre os diversos queijos artesanais (HARBUTT, 2009).

Dentre os queijos produzidos com leite cru destacam-se os queijos europeus franceses (Roquefort, Camembert e Brie), italianos (Parmigiano-Reggiano, Mussarela, Provolone, Grana Padano, Pecorino romano, Fiore Sardo e Canestrato Pugliese),

espanhóis (Manchego, Majorero, Cabreiro e Cabrales), sendo que a maioria possui denominação de origem protegida (ALBUQUERQUE, 2003; DI CAGNO, 2003; HARBUTT, 2009; MASUI; YAMADA, 1999). No Brasil, por sua vez, destacam-se os queijos tipo Minas produzidos nas regiões de Araxá, Serra da Canastra, Alto Paranaíba e Serro, os queijos Manteiga e Coalho, produzidos no Nordeste e os queijos Colonial e Serrano, provenientes do Sul. Há poucas e limitadas informações sobre estes produtos artesanais/coloniais, porém, sabe-se que os mesmos apresentam grande importância social, econômica e cultural (PINTO, 2004).

A produção nacional de queijos aumentou nas últimas décadas, o que refletiu-se num aumento do consumo per capita do mesmo, que atualmente atinje 4 Kg/habitante ao ano (SEBRAE, 2014). Embora a maior parte da produção nacional seja referente a queijos produzidos em indústrias, muitas variedades podem ser classificadas como produtos artesanais, sendo a maior parte (40%) da produção comercializada de maneira informal (SEBRAE, 2008).

A técnica de elaboração artesanal de queijos no Brasil iniciou-se por meio dos colonizadores portugueses, que nos primeiros anos da colônia introduziram o gado bovino e passaram a produzir o queijo português de Serra da Estrela, o qual deu origem aos típicos queijos artesanais (REIS, 1998). Estes queijos tradicionais são fabricados manualmente em fazendas ou vilarejos há mais de 200 anos e apresentam uma forte ligação com o território de origem, testemunhando a história, a cultura e o estilo de vida das comunidades produtoras, o que se reflete na intensidade e diversidade de sabores típicos de cada região (LICITRA, 2010).

Uma ação importante que contribuiu para o reconhecimento e a afirmação da tradição dos queijos artesanais, foi o título de Patrimônio Cultural do Brasil conferido pelo

Instituto do Patrimônio Histórico e Artístico Nacional (IPHAN) em 2006 (NÓBREGA, 2012). Algumas regiões do Brasil apresentam regulamentações locais da produção de queijos:

- Minas Gerais, em 2011, definiu uma certificação diferenciada para os queijos fabricados conforme a tradição histórica e cultural da região produtora com a regulamentação da Lei 19.492 (MINAS GERAIS, 2011), que alterou a Lei n° 14.185, de 31 de janeiro de 2002;

- No nordeste, ambos os queijos coalho e manteiga são regulamentados pela Instrução Normativa nº 30, de 26 de junho de 2001, do Ministério da Agricultura, Pecuária e Abastecimento (BRASIL, 2001);

- Na Região Sul, a identidade e a qualidade do queijo Serrano são regulamentadas pela Portaria nº 214, de 14 de dezembro de 2010 (RIO GRANDE DO SUL, 2010), já para o queijo colonial não há legislação específica que regulamente o seu Padrão de Identidade e Qualidade (BRASIL, 1996);

- No Mato Grosso do Sul, a produção do queijo caipira é regulamentada desde 2004, com a lei nº 2.820, de 04 de maio de 2004, sugerindo o estabelecimento desse produto e sua tradição (NÓBREGA, 2012).

A microbiota endógena particular (associada à região de origem e à tecnologia de fabricação) confere aos queijos artesanais características organolépticas peculiares e é composta principalmente por bactérias láticas e leveduras presentes no leite e no ambiente de processamento. Estes micro-organismos também estão presentes como cultura endógena denominada “pingo”, que consiste em uma porção de soro fermentado originado do dessoramento de queijos produzidos no dia anterior, coletado em vasilhames para ser utilizado como fermento na produção do dia posterior (LIMA et al.,

2009; LEITE, 1993). Porém, há uma redução brusca desta população quando o leite é submetido à pasteurização ou tratamento térmico equivalente, o que influencia negativamente no desenvolvimento das características sensoriais do queijo, como a menor intensidade no aroma, devido à redução na quantidade de oligopeptídeos e aminoácidos livres, componentes imprescindíveis para a extensão da proteólise, principal modificação bioquímica dos queijos durante a maturação (MAARA, 1996; GRAPPIN; BEUVIER, 1997). Em razão disto, a adição de fermento lático comercial na elaboração de queijos, produzidos a partir de leite pasteurizado, tem ocasionado mudanças nas suas características sensoriais e perda da identidade destes produtos artesanais (ESTEPAR et al., 1999).

Com a publicação da Instrução Normativa nº 30, de 07 de Agosto de 2013, que preconiza que outros órgãos estaduais e municipais possam avaliar a certificação de produtos artesanais, tornou-se possível ao produtor de pequena propriedade rural, a comercialização de seus queijos em todos os estados, desde que este tenha maturação superior a 60 dias ou inferior (quando respaldado por estudos científicos que garantam a inocuidade deste alimento) e esteja de acordo com premissas estabelecidas pela legislação (BRASIL,2013). Porém, esta legislação se contrapõe ao processo histórico de fabricação dos queijos artesanais de Minas das regiões das Serras da Canastra, Serro e Salitre, por exemplo, que desde o período colonial são maturados em torno de 20 dias, sendo incapaz de resistir ao período de quarentena pré-estabelecido sem deteriorar-se (CERRI, 2002). Por exemplo, sabe-se que a maturação dos queijos por até 60 dias modificou a textura e as suas características organolépticas, com perda de umidade, aumento da concentração de sal e do grau de proteólise, resultando em um produto com menor aceitação sensorial (LIMA et. al, 2008). Além disso, sabe-se que parâmetros como

a qualidade da água, da cultura endógena empregada, aplicação das boas práticas de fabricação durante o processamento do queijo, variam entre os estabelecimentos, e desta forma, o tempo de maturação pode ser variável (MARTINS et al., 2015).

Neste contexto, o isolamento de micro-organismos naturalmente presentes na microbiota de queijos artesanais e o estudo de suas propriedades tecnológicas, como produção de ácido lático, produção de sabor e aroma desejáveis durante a cura, capacidade de acelerar a cura, tolerância ao sal, produção de bacteriocinas, resistência a bacteriófagos, dentre outras (AYAD et al., 2004; FOX et al., 2000; HASSAN; FRANK, 2001) constitui-se em um importante passo para obtenção de culturas microbianas visando-se obter características e, ao mesmo tempo, melhorar a qualidade e padronização dos queijos artesanais (CARVALHO et al., 2005; CARVALHO, 2007). Um produto padronizado e de alta qualidade, pode alcançar mercados diversos, no âmbito nacional e internacional, e desta forma representar importantes fontes de divisas de valor agregado para o setor de agricultura brasileiro.

Pelo exposto anteriormente, fica patente a grande relevância das culturas microbianas (iniciadoras ou não) para o desenvolvimento e melhoria da qualidade de alimentos, principalmente fermentados. Para aplicação no processamento de alimentos, as culturas microbianas devem possuir algumas características desejáveis que incluem rápido crescimento, alto rendimento em biomassa e em produto e algumas propriedades organolépticas específicas (SMID, 2014). No entanto, sabe-se que há dificuldades inerentes ao processo de seleção de cepas para atuarem como culturas microbianas em processos biotecnológicos aplicados à alimentos. Por exemplo, algumas cepas podem possuir propriedades antimicrobianas adequadas, mas não necessariamente serem competitivas em um processo industrial. Outras podem ser altamente competitivas em

processos industriais, mas carrearem genes de virulência. Desta forma, o melhoramento de cepas visando-se alcançar fenótipos desejáveis constitui-se em uma excelente alternativa, sendo conhecido como "engenharia evolutiva" (BACHMANN, 2015).

A engenharia evolutiva imita os processos evolutivos naturais, que consistem em ciclos iterativos de diversificação genética e seleção funcional ou *screening*, e ao contrário dos métodos “racionais”, é menos dependente do conhecimento prévio da relação genótipo-fenótipo. A cepa melhorada é obtida através da diversidade genética criada de maneira eficiente por mutagênese (natural ou induzida) e por recombinação ou rearranjo de genes, vias e genomas, seguido da seleção por alto rendimento ou um fenótipo desejado. As cepas utilizadas nos processos industriais são frequentemente obrigadas a possuir vários fenótipos, tais como: tolerância a produtos metabólicos, inibidores e maior produtividade, a fim de satisfazer os critérios de comercialização. A engenharia evolutiva pode ser utlizada com este propósito, pela evolução e identificação de cepas adaptadas utilizando critérios de seleção que refletem as condições de processo, ou seja, uma pressão seletiva que age como força motriz para a seleção de mutantes com fenótipos aprimorados (KIM et al., 2012; PAIVA; RUSSELL, 1999; UKIBE et al., 2009). Devido aos curtos tempos de geração e ao grande tamanho das populações, a adaptação evolutiva de micro-organismos a estas pressões de seleção impostas experimentalmente podem ser facilmente estudadas em laboratório (BARRICK; LENSKI, 2013). Além disso, outra vantagem da aplicação desta ferramenta é que as cepas obtidas por engenheria evolutiva não são consideradas como geneticamente modificadas, o que aumenta a aceitação do mercado consumidor.

A engenharia evolutiva vem sendo amplamente aplicada em processos biotecnológicos visando a produção de biocombustíveis, cerveja e vinho. Por exemplo,

Ekberg et al. (2013), ao cultivar leveduras de cerveja sob condições condições hiperosmóticas, obtiveram melhoria na capacidade de fermentação em mosto a altas pressões; enquanto que Tilloy et al. (2014), em condições semelhantes, obtiveram leveduras de vinho com reduzida produção de etanol e maior produção de glicerol; Bachmann et al. (2012), ao submeterem uma cepa de *Lactococcos lactis* isolada de plantas a um cultivo descontínuo em série em leite durante 1000 gerações, obtiveram um aumento de 50% na taxa específica de acidificação em relação à cepa ancestral, mostrando ser possível "domesticar" isolados da natureza por meio de engenharia evolutiva. Na indústria vinícola, Cadière et al. (2011) e Cadière et al. (2012) obtiveram leveduras de vinho com características melhoradas em fermentações de escala laboratorial, como: maior taxa de fermentação, diminuição na formação de acetato e maior produção de compostos aromáticos, ao utilizar a engenharia evolutiva baseada no cultivo descontínuo em série em meio contendo gluconato como única fonte de carbono. Apesar disso, observa-se que há ainda um enorme potencial para aplicações da engenharia evolutiva em processos biotecnológicos aplicados à alimentos, como na obtenção de culturas adjuntas para aplicação em queijos, visando-se reduzir o tempo de maturação.

Considerando-se o potencial da microbiota endógena dos queijos artesanais para aplicação biotecnológica, como produção de enzimas (proteolíticas, lipolíticas e glicolíticas) essenciais para o processo de maturação dos queijos e resistência às condições de processamento, a presente tese teve como objetivo estudar as propriedades tecnológicas e probióticas de bactérias láticas selvagens, associando tais conhecimentos à engenharia evolutiva para a obtenção de uma cultura e/ou mistura de culturas com cepas mais robustas (mais resistentes e adaptadas), capazes de melhorar

a performance durante a fermentação, e após submissão às condições simuladas do TGI. Trata-se de uma estratégia que permitirá o aproveitamento de recursos de biodiversidade de nosso país, para manter características regionais, assegurar a manutenção do patrimônio cultural, social e econômico associado aos queijos artesanais. Além disso, será possível gerar *know-how* (licenciamento das culturas desenvolvidas) e tecnologia para sua aplicação no processamento de queijos artesanais.

REVISÃO DE LITERATURA

1. Bactérias láticas e sua importância em queijos

Historicamente, a utilização de bactérias láticas (BAL) em produtos lácteos é bastante difundida. Muitos são os benefícios que esses micro-organismos podem conferir aos alimentos, como a conservação de suas propriedades nutricionais, o incremento no sabor e a capacidade de conferir maior segurança microbiológica (COSTA et al., 2007). Este grupo de bactérias é formado por micro-organismos Gram-positivos, catalase negativos, não formadores de esporos e que geralmente crescem sob condições microaerófilas ou estritamente anaeróbicas.

Os gêneros de BAL mais comumente encontrados em alimentos são: *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc* e *Enterococcus* (FOX; MCSWEENEY, 2000; BERESFORD et al., 2001). Estas bactérias também podem ser agrupadas em espécies homofermentativas, quando produzem ácido lático como principal produto de fermentação, ou heterofermentativas, quando produzem, além de ácido lático, dióxido de carbono, ácido acético, etanol, aldeído e diacetil (CARR et al.,

2002). Em relação à temperatura de multiplicação, elas podem ser classificadas em mesofílicas e termofílicas, cujas temperaturas ótimas de multiplicação são por volta de 30 °C e 42 °C, respectivamente (FOX et al., 2000).

Na fabricação da maioria dos queijos, cepas de BAL são criteriosamente selecionadas e adicionadas ao leite pouco antes da adição do coalho, com a função principal de produzir ácido lático, compostos aromatizantes, como acetaldeído e diacetil (FOX, 2000). As BAL iniciadoras ou *starters* devem fermentar rapidamente a lactose, produzindo elevadas concentrações de ácido lático a fim de reduzir rapidamente o pH do leite, que, usualmente, deve chegar a valores menores que 5,3 em 6 horas a 30-37 ºC, dependendo da variedade do queijo (FOX et al., 2004; COGAN et al., 1997). Fazem parte deste grupo, *Lactococcus lactis* e *Leuconostoc* spp., entre cepas mesofílicas e *Streptococcus thermophilus*, *Lactobacillus delbrueckii* e *Lactobacillus helveticus* entre cepas termofílicas (FOX et al., 2004).

O queijo constitui-se em um ambiente hostil para a multiplicação da maioria dos micro-organismos durante a maturação, devido à presença de sal, baixa umidade, baixos valores de pH (4,9 e 5,3), baixas temperaturas e limitação de nutrientes (TURNER et al., 1986). Porém, uma grande diversidade de micro-organismos, incluindo bactérias, leveduras e bolores podem estar presentes nos queijos durante a etapa de maturação e contribuir positivamente para este processo. Esta contribuição se dá, tanto pela atividade metabólica direta, quanto pela liberação de enzimas na matriz de queijo por autólise, as quais podem exercer muitos efeitos benéficos sobre as características sensoriais do queijo (PETERSON; MARSHALL, 1990).

Os grupos microbianos normalmente presentes nos queijos incluem: (i) bactérias láticas não iniciadoras (*Non-starter lactic acid bacteria* - *NSLAB*), constituído de

Lactobacillus não iniciadores, *Pediococcus*, *Enterococcus* e *Leuconostoc*; (ii) bactérias propiônicas; (iii) bolores e (iv) bactérias e leveduras que crescem sobre a superfície de queijos curados. Tal divisão sugere que o leite não é a principal fonte de *NSLAB* em queijos feitos com leite pasteurizado, mas sim os equipamentos, ambiente, manipuladores, etc. (MARTLEY; CROW, 1993). Sabe-se que as "*non-starter* lactic acid bacteria" (*NSLAB*) desempenham papel fundamental na maturação de queijos, sendo que as espécies de *NSLAB* mais comumente isoladas durante a maturação do queijo são: *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Enterococcus durans*, *Enterococcus faecalis*, *Enterococcus faecium* e *Leuconostoc* (CHAMBA; IRLINGER, 2004).

O aroma característico de diversos queijos maturados resulta primordialmente das reações de lipólise e proteólise, em parte considerável, provenientes da ação das *NSLAB*. A proteólise é o principal e mais complexo evento bioquímico que ocorre durante a maturação da maioria das variedades de queijos, dando origem a numerosos produtos, como peptídeos, cetonas e aminoácidos livres, que irão garantir o sabor, aroma e textura característicos dos queijos, devido a atuação de várias enzimas envolvidas no processo, principalmente as microbianas (GUTIERREZ, 2004; SIHUFE et al., 2005). Os perfis de compostos voláteis gerados são específicos para cada isolado de BAL e/ou suas combinações. Mauriello et al. (2001), ao comparar a produção de compostos voláteis neutros no soro de leite, mostraram que a produção de compostos aromáticos se mostrou variável entre *Lactococcus*, *Streptococcus*, *Enterococcus*, *Lactobacillus* mesófilos e termofílicos, e, em alguns casos entre as cepas. Ayad et al. (1999) salientaram que *Lactococcus* selvagens isolados a partir de produtos de origem láctea ou não-láctea produzem aromas distintos dos isolados industriais. Logo, a seleção e utilização de novos

isolados de BAL capazes de formar sabores e aromas específicos é considerada uma abordagem promissora para responder à crescente demanda por queijos com propriedades organolépticas melhoradas.

Além de atuarem na melhoria de sabor e aroma dos queijos, as *NSLAB* podem ser candidatas à elaboração de novas culturas lácteas para produção de produtos lácteos fermentados potencialmente funcionais, podendo auxiliar na segurança destes produtos e na preservação da microbiota original de queijos artesanais (COSTA et al., 2013).

2. Probióticos

Além das propriedades tecnológicas, algumas cepas de BAL e leveduras podem apresentar propriedades probióticas. De acordo com a Organização das Nações Unidas para a Agricultura e Alimentação, probióticos são definidos como “micro-organismos vivos que, quando administrados em quantidades adequadas, conferem benefícios à saúde do hospedeiro” (FAO/WHO, 2002), inibindo o crescimento de micro-organismos patogênicos pela competição por substratos; impedindo a aderência de bactérias patogênicas nas células hospedeiras do intestino, reforçando o efeito de barreira da mucosa intestinal (EIZAGUIRRE et al. 2002;. MANGELL et al. 2002.); liberando metabólitos que protegem o intestino (arginina, glutamina, ácidos graxos de cadeia curta e ácidos linoléicos conjugados) e, por fim, secretando compostos antimicrobianos, como bacteriocinas e substâncias, tais como ácidos orgânicos (ácido lático, ácido acético e ácido butírico) e peróxido de hidrogênio (H_2O_2).

Por apresentarem diferentes mecanismos de ação, leveduras e bactérias apresentam comportamento sinérgico e maior viabilidade quando em co-cultura (BISSON

et al., 2010; SUHARJA et al., 2012), pois as leveduras interagem positivamente com bactérias probióticas, aumentando sua sobrevivência e estimulando seu crescimento (GOBBETTI et al.,1994; LIUANDTSAO, 2009; KATAKURA et al.,2010; SUHARJA et al., 2012) devido à produção de nutrientes, como peptídeos, aminoácidos e vitaminas (GOBBETTI et al., 1994; VILJOEN, 2001; NARVHUS; GADAGA, 2003; KAWARAI et al., 2007; KATAKURA et al., 2010). Até o presente momento, apenas a levedura *Saccharomyces boulardii* teve atividade probiótica comprovada por estudos, podendo ser consumida por humanos (VANDERAA KÜHLE et al., 2005). Esta levedura é usada em muitos países como agente preventivo e terapêutico contra diarréia e outras disordens do trato gastrointestinal causados pela ação de antibióticos administrados inadequadamente. Porém, algumas espécies de leveduras como *D. hansenii, Torulaspora delbrueckii* (PSANI; KOTZEKIDOU, 2006), *Kluyveromyces lactis*, *Yarrowia lipolytica* (CHEN et al., 2010), *K. marxianus*, *K. lodderae* (KUMURA et al., 2004) têm mostrado forte atividade antagonista frente a bactérias patogênicas e resistência à passagem pelo trato gastrointestinal.

Os micro-organismos probióticos podem ser veiculados à dieta humana através de diversas matrizes alimentícias. No entanto, sabe-se que produtos lácteos, e principalmente os queijos, são produtos alimentares amplamente disponíveis hoje em dia, atraentes para muitos paladares e apropriados para todas as faixas etárias. Estes aspectos oferecem oportunidades para muitas estratégias de marketing relacionadas ao consumo dos queijos (WILKINSON, MEEHAN, STANTON, & COWAN, 2001) e excelente matriz para veiculação dos probióticos. No entanto, o desenvolvimento de queijos probióticos implica o conhecimento obrigatório de todas as etapas de processamento, bem como do nível de influência (positivo ou negativo) de cada uma na sobrevivência

desses micro-organismos durante toda a vida de prateleira do produto, de maneira que as células estejam viáveis em benefício do consumidor (CRUZ et al., 2009).

Além dos aspectos mencionados anteriormente, assim como em qualquer alimento probiótico, para exercer estes benefícios para a saúde do consumidor, as bactérias probióticas incorporadas no queijo devem ser capazes de se multiplicarem no intestino humano. Além disso, elas devem também ser capazes de sobreviver durante a passagem através do trato gastrointestinal (TGI), o que envolve a exposição ao ácido clorídrico e sais biliares, no estômago e intestino delgado, respectivamente (STANTON et al., 2003). Deste modo, um conjunto de critérios relevantes devem ser atendidos para que um micro-organismo seja considerado probiótico: (i) ser identificado à nível de gênero, espécie e linhagem, (ii) ser capaz de produzir substâncias antimicrobianas (por exemplo, bacteriocinas), (iii) ser seguro para o uso em alimento e clínico, (iv) ser capaz de sobreviver a passagem pelo TGI, (v) ser capaz de aderir a superfícies mucosas, (vi) ser capaz de colonizar o intestino humano (pelo menos temporariamente), (vii) ser capaz de inibir bactérias patogênicas, (viii) possuir efeito benéfico à saúde clinicamente documentado e validado (ix) ser estável durante o processamento e armazenamento dos alimentos (VERNA; LUCAK, 2010; SAAD et al. 2013; FAO, 2002).

3. O processamento de queijos – enfoque na etapa de maturação

Em queijos artesanais brasileiros, a etapa de maturação é essencial para a eliminação ou diminuição das populações de micro-organismos patogênicos e deteriorantes potencialmente presentes, pois consiste em uma série de processos físicos,

químicos e microbiológicos que desfavorecem o desenvolvimento destas bactérias. A produção de queijo é pautada na coagulação enzimática do leite e nas reações que estão envolvidas na maturação, que seguem por três vias bioquímicas principais: proteólise, lipólise e glicólise, responsáveis pelas mudanças fisico-químicas e sensoriais observadas em queijos maturados, como o desenvolvimento de cor, textura e aroma. A *Figura 1* resume as principais vias metabólicas envolvidas no processo de maturação dos queijos e os principais produtos formados. As enzimas que regem estas reações são principalmente: a quimosina (renina) ou substitutos de renina, enzimas provenientes do próprio leite, de culturas iniciadoras eventuais e de culturas não iniciadoras. Outros fatores como tempo, pH, concentração de sal e temperatura também afetam o processo de maturação (SOUSA et al., 2004; COLLINS et al., 2003ª; FOX et al., 1996; PAULSON et al., 1998).

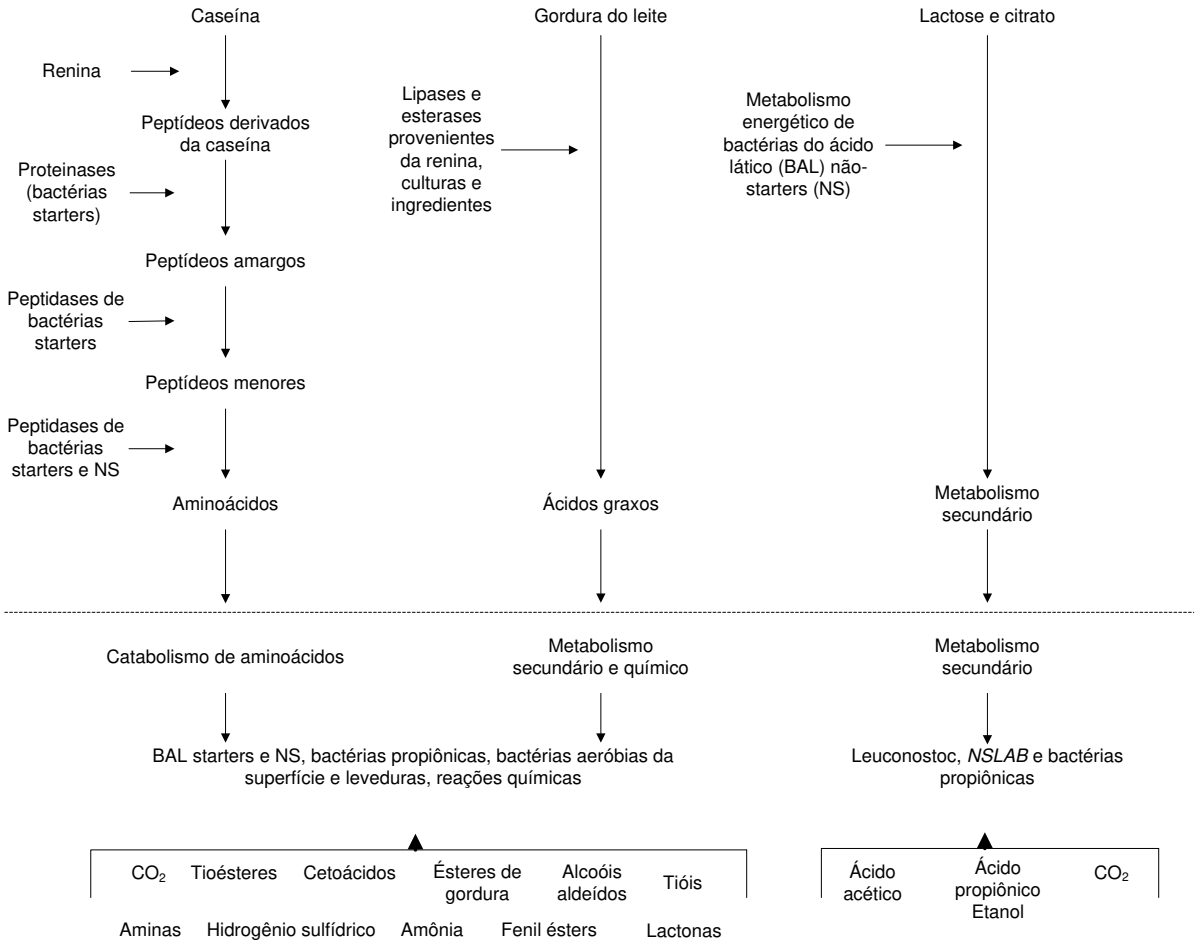

Figura 1. Bioquímica básica envolvida na maturação de queijos (LAW, 2005).

Aminas, aldeídos, álcoois e amônia, derivados da degradação de aminoácidos são os principais compostos envolvidos na formação de compostos aromáticos dos queijos. Além disso, a quebra da rede proteica provoca alterações texturais na matriz dos queijos (SOUSA et al., 2001). A lipólise, também importante no desenvolvimento de sabor de queijo, (FORDE; FITZ-GERALD, 2000; COLLINS et al.,2003) resulta na formação de ácidos graxos livres a partir da hidrólise dos triglicerídeos. Os compostos voláteis, tais como cetonas de metila, tioésteres e lactonas, resultam do catabolismo destes ácidos graxos livres (SOUSA et al, 2001; COLLINS et al., 2003). Outros compostos, como

diacetil, ácido acético e propiônico, são derivados de glicólise (FORDE; FITZ-GERALD, 2000).

Segundo SOUZA, ARDO e McSWEENEY (2001), a proteólise durante a maturação é influênciada pelo sistema de proteinases e peptidases do coagulante residual, das enzimas endógenas do leite, do fermento lático, BAL que não fazem parte do fermento, e culturas láticas secundárias, como por exemplo, bactérias propiônicas, *Brevibacterium*, *Arthobacter*, *Penicillium* ssp., além da razão sal/umidade, temperatura de maturação e modificação do pH durante a maturação.

Segundo WOLFSHOON-POMBO (1983), as mudanças durante a maturação são designadas pela “extensão” e “profundidade” da proteólise. O índice de extensão de maturação (IEM) é resultante da ação proteolítica das enzimas do coalho sobre a caseína do queijo. A ação destas enzimas tem como resultado a liberação de peptídeos de alto peso molecular, caracterizando-se pela quantidade de substâncias solúveis na fase aquosa dos queijos. A liberação de tais peptídeos constitui-se em um fator de grande importância na composição final e na qualidade do produto (WOLFSCHOON-POMBO; LIMA, 1989). O índice de profundidade de maturação (IPM), por sua vez, tem a finalidade de verificar a formação de substâncias de baixo peso molecular acumuladas durante o período de maturação, principalmente por causa da ação proteolítica das enzimas microbianas sobre compostos nitrogenados oriundos da degradação da caseína. Compostos característicos dessa degradação são os aminoácidos, oligopeptídeos e aminas (SILVA et al., 1995).

Sabe-se que a etapa de maturação, apesar de crucial para as características organolépticas dos diversos tipos de queijo, possui um custo elevado, principalmente associado ao tempo de estocagem sob condições controladas de temperatura. Assim,

têm-se desenvolvido novos métodos para acelerar a maturação de queijos a fim de aumentar a produção, como: aumento da temperatura durante o período de maturação, uso de enzimas exógenas, emprego de culturas atenuadas e/ou adjuntas, emprego de culturas iniciadoras geneticamente modificadas ou com enzimas recombinantes e a microencapsulação de enzimas da maturação (proteinases, peptidases, lipases e β-galactosidase). Porém, tais métodos apresentam algumas desvantagens, como: baixa disponibilidade de preparo de enzimas comerciais, dificuldade em distribuir uniformemente as mesmas na matriz do queijo, além do alto custo associado e/ou baixa eficiência, no caso da microencapsulação ou uso de lipossomas (AZARNIA, 2006).

Dentre os métodos anteriormente descritos, a elevação da temperatura de maturação consiste no mais efetivo, simples e menos custoso para acelerar a maturação. Tal método pode ser aplicado através da elevação e manutenção da temperatura de maturação durante todo o período em que esta etapa ocorre, ou utilizando-se diferentes combinações de temperatura-tempo (isto é, a utilização de temperaturas de armazenamento elevadas durante um período limitado combinada com temperaturas baixas habituais para o resto do período de maturação). Apesar disto, considerando-se as numerosas e complexas reações bioquímicas que ocorrem durante a maturação, é pouco provável que todos os processos serão igualmente acelerados. Desta forma, desequilíbrio de sabores ou sabores não característicos podem ser desenvolvidos, além do potencial crescimento de micro-organismos patogênicos (HANNON et al. 2005; FOX et al., 1996). Neste contexto, a engenharia evolutiva surge como uma ferramenta alternativa e eficaz para o melhoramento de cepas e, consequentemente, do processo de fermentação e maturação que rege a produção queijeira.

4. Engenharia evolutiva como método alternativo para acelerar a maturação

O melhoramento de linhagens através da engenharia evolutiva baseia-se no princípio básico da variação genética (natural e/ou induzida). Geralmente, uma população de células é cultivada sob seleção por muitas gerações (divisões celulares) e, por meio do uso de uma pressão seletiva como força motriz (PAIVA; RUSSELL, 1999; UKIBE et al., 2009). Com o passar do tempo, os mutantes aleatórios surgirão nesta população (STEENSELS et al., 2014) e podem ou não ter características mais relevantes que seus iniciadores. Devido ao curto tempo de geração, a manipulação e cultivo de micro-organismos no laboratório pela engenharia evolutiva, é um caminho possível para gerar cepas microbianas com fenótipos melhorados (ELENA; LENSKI, 2003).

O desenvolvimento de estirpes microbianas industrialmente relevantes é uma tarefa difícil, devido à complexidade das células microbianas e dos fenótipos requeridos para os processos industriais. Abordagens racionais para melhoria de cepas microbianas envolvem a inserção ou retirada de genes-alvo específicos nos cromossomos. No entanto, os fenótipos complexos associados com múltiplos genes e suas interações são difíceis de serem alcançados por desenho racional tendo como alvo um ou alguns genes de uma só vez. Embora o avanço de ferramentas analíticas nas áreas de genômica, proteômica e metabolômica tenham reduzido significativamente o trabalho, o tempo e os custos associados com a engenharia das cepas, o sucesso de abordagens racionais depende largamente da compreensão detalhada das redes bioquímicas e reguladoras (WARNER et al., 2009), ao contrário da engenharia evolutiva. O surgimento destas

ferramentas de biologia molecular deu-se na década de 1980 e levou a uma mudança no foco de pesquisa para a engenharia metabólica. No entanto, a má aceitação do consumidor de organismos geneticamente modificados (OGM) nos produtos alimentícios estimulou um renascimento de métodos não-OGM, tais como seleção de cepas e evolução laboratorial de micro-organismos utilizados no processamento dos alimentos (BACHMAN et al., 2015).

O conceito básico da engenharia evolutiva é também evidenciado no desenvolvimento clássico e empírico de cepas por mutagênese clássica aleatória e por seleção direta em placas. Esta abordagem teve uma longa história de sucesso no desenvolvimento de cepas industriais, particularmente com ausência de extensa informação genética ou fisiológica. O melhor exemplo disso é, provavelmente, o melhoramento de 4.000 vezes na produção de penicilina, por esta abordagem (PAREKH et al., 2000; ROWLANDS, 1984; VINCI; BYNG, 1999). Este método também tem sido reconhecido como uma estratégia promissora na engenharia de proteínas. A engenharia evolutiva tem sido empregada com sucesso para melhorar a função de várias proteínas em milhares de vezes e também para construção de novas propriedades enzimáticas artificiais, não encontradas em ambiente natural (MINSHULL; STEMMER, 1999; ARNOLD; VOLKOV, 1999). O controle da atividade das peptidases de BAL da microflora de queijos artesanais é, por conseguinte, um fator chave na tecnologia de maturação, que tem sido alcançado, em parte, pela tecnologia de enzimas e por modificações genéticas. A engenharia evolutiva tem grande potencial para aplicação neste âmbito, pois não envolve micro-organismos geneticamente modificados e é mais barata (LAW, 2001).

A engenharia evolutiva baseia-se no princípio de que as populações de células podem se adaptar ao seu ambiente ao longo do tempo pela seleção natural. Esta

abordagem foi utilizada para criar leveduras mutantes que são tolerantes a vários fatores de estresse (STANLEY, 2010), tais como o congelamento/descongelamento (TAKAGI, 1997), temperaturas elevadas (WATANABE, 2009), elevadas concentrações de sal (MA, 2010) e elevadas concentrações de ácido acético (AMORIM, 2004).

A engenharia evolutiva (evolução adaptativa) pode ser realizada em modo descontínuo ou em culturas contínuas. Em culturas contínuas, as variantes com melhor aptidão para um determinado ambiente se sobressaem ao longo do tempo e substituem a população parental. Na cultura descontínua ou em “batch”, uma pequena fração (geralmente 10%) da cultura corrente é transferida para novo substrato antes de haver a depleção de nutrientes, e este processo é repetido sequencialmente até que o número alvo de gerações seja alcançado. As células de cada cultura em “batch” passam pelas fases lag, exponencial e fase estacionária de crescimento, sendo que em cada ciclo há uma mudança significativa nas condições seletivas de crescimento, de ambientes ricos em nutrientes para ambientes limitados em nutrientes, por exemplo. Nas culturas em batelada sequenciais, verificou-se que houve uma melhora da aptidão das cepas nas primeiras fases de experimentos de evolução adaptativa, e a taxa de melhoria da aptidão competitiva diminuiu hiperbolicamente ao longo do tempo (KIM, 2012). O grande desafio experimental nestes métodos de engenharia evolutiva consiste em ligar uma característica específica e relevante industrialmente com o aumento da taxa de crescimento específico e/ou sobrevivência de células mutantes individuais (BARRICK; LENSKI, 2013; SAUER, 2001). Se as mutações individuais causam uma grande e suficiente vantagem seletiva, os mutantes podem ser enriquecidos e isolados numa etapa única de cultivo descontínuo (SOLOPOVA et al., 2012; MARGOLLES; SÁNCHEZ, 2012).

No entanto, o cultivo prolongado é normalmente usado para permitir a seleção por mutações sucessivas que conferem um incremento na vantagem seletiva.

O cultivo descontínuo em série, que seleciona mutantes com taxa de crescimento específica máxima maiores quando a transferência entre meios ocorre na fase exponencial, pode ser realizado em culturas em balão simples ou em reatores em bateladas sequenciais (RBSs) automatizados. Os RBSs devem ser cuidadosamente desenhados para evitar a seleção não intencional de fenótipos de rápida sedimentação durante o ciclo de preenchimento e extração (Oud et al., 2013). Devido aos seus curtos tempos de geração, as bactérias são bem adequadas para a investigação de estratégias evolutivas em ambiente laboratorial. A evolução experimental de *Escherichia coli* por 40.000 gerações em meio limitado em glicose é um dos exemplos mais conhecidos na área, a fim de acompanhar as mutações benéficas em seu genoma (BARRICK et al. 2009).

A engenharia evolutiva pode também permitir a investigação imparcial dos fatores genéticos que levam a melhoria do desempenho microbiano em ambientes altamente complexos. Por exemplo, para os *Lactobacillus* que são comercializados como probióticos, o aumento do tempo de residência no trato gastrointestinal (TGI) é bastante desejável. A permanência no TGI foi recentemente considerada ser altamente cepa-específica (VAN et al., 2012) e as condições de crescimento *in vitro* afetam fortemente a sobrevivência e a persistência de culturas bacterianas em condições intestinais (BRON et al., 2012). Notavelmente, a exposição repetitiva de *Lactobacillus plantarum* ao trato intestinal de ratos por re-alimentação consecutiva das colônias com maior persistência, permitiu o isolamento independente de derivados adaptados ao intestino provenientes da cepa original, com um tempo de residência no TGI cinco vezes maior.

A aplicação da engenharia evolutiva (ou evolução adaptativa) para obtenção e melhoramento de culturas adjuntas e/ou iniciadoras para aplicação na indústria queijeira apresenta-se como uma estratégia inovadora e extremamente atual. As principais vantagens da aplicação da engenharia evolutiva neste caso seriam um potencial aumento da produção de compostos aromáticos e a aceleração das reações envolvidas na maturação dos queijos, culminando com a redução do tempo em que esta etapa ocorre e com uma padronização do sabor, odor e textura dos produtos finais.

CAPÍTULO 1: Brazilian artisanal cheeses as a potential source of wild lactic acid bacteria to be applied in dairy industry. Part I: High throughput screening of technological and probiotic properties

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Brazilian artisanal cheeses as a potential source of wild lactic acid bacteria to be applied in dairy industry. Part I: High throughput screening of technological and probiotic properties

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Abstract

Brazilian artisanal cheeses are a great source of wild lactic acid bacteria (LAB) with interesting properties to be industrially explored. Such properties vary among species, according to their source of isolation, which justifies differences in the peculiar flavor inherent to each cheese and their regions. This study aimed to evaluate technological (acidification, proteolysis, lipolysis, resistance to low pH, NaCl and bile salts, at ranged levels) and bio preservation (bacteriogenic activity against foodborne pathogens) features of 1,002 LAB, isolated from 11 types of Brazilian artisanal cheeses, marketed in the main 5 regions and validated their potential for application in the dairy industry as starter and/or adjunct cultures. There was a great variability intra-species in acidification rates, mostly between isolates from Araxá, Campo das Vertentes, Caipira, Coalho and Serro cheeses, with *Lactobacillus paracasei* and *Lactobacillus brevis* showing the best performance, but all isolates with low rates when compared to the control (4.3x lower), being considered as Non-starter cultures. Nine strains identified as *Lactobacillus plantarum* and *Lactobacillus buchneri* were able to inhibit *Listeria monocytogenes* growth using its sterile supernatants. Principal component analysis was assayed in order to verify clusters, relating studied variables and origin of strains. It was possible to verify different groups formed by high proteolytic and lipolytic strains; inhibitors of *L. monocytogenes* and high resistance to salt stress. The highlight of this study was to set a multivariate high-throughput screening in order to obtain insights related to the favourable combination of functional and technological properties of LAB and to select them for further application as starters in fermented food. *Lb. plantarum* was highly isolated from Brazilian Artisanal Cheeses and showed a large intraspecific heterogeneity. The type of cheese influenced some properties analysed, such as the production of bacteriogenic compounds bacteriocins, mostly produced by *Lb. plantarum* and *Lb. buchneri* from Minas Artisanal Cheeses; and the production of lipolytic enzymes by *Pediococcus acidilactis* from Marajó cheese. In general, the strains presented a good potential to be applied as biopreservatives, probiotics and as adjuncts cultures, contributing to flavor and texture during ripening.

Keywords: Brazilian artisanal cheese, lactic acid bacteria, adjunct cultures, biotechnology, food quality, food safety.

1. Introduction

Brazilian artisanal cheeses are made from different types of raw milk, such as cow (Artisanal Minas, Colonial, Caipira and Serrano cheeses), buffalo (Marajó) or goats (Coalho and Manteiga cheeses). Generally, they are produced without using industrial starter cultures, except for some traditional producers from Minas Gerais state who use a natural culture obtained by whey fermentation, called “pingo” through a procedure called back-slopping (Kamimura et al., 2019). These cheeses present an important historical, social, economic and cultural role for traditional communities, who have been passing down the way to produce artisanal cheeses for over 200 years (A. V. Silva et al. 2014; Silva et al., 2012; A. G. Cruz et al. 2009; Arcuri et al. 2013; Borelli et al. 2006; Cardoso et al. 2013; Carlos Gonçalves Costa Júnior et al. 2014; Cruz & Menasche, 2014; Funck et al. 2015). An important action that contributed to the recognition and affirmation of traditional artisanal cheeses in Brazil was the title of Cultural Patrimony of Brazil conferred by the National Historical and Artistic Heritage Institute (IPHAN) in 2006 (Nóbrega, 2012). Besides that, they still represent 40% percent of the national production, but in an informal way (without inspection) (Sebrae, 2008).

Recently, Brazilian legislation recommended to use pasteurized milk in dairy products, and, for artisanal cheese production (which implies raw milk usage), the ripening time should not be less than 60 days unless it is proven that shorter ripening times are safe. This should guarantee standardization and quality control during the process, increasing consumers health security. However, a concern is that artisanal cheeses might lose their characteristic features because pasteurization of milk may destroy the endogenous microbiota present in raw milk. These endogenous organisms are mostly

represented by Lactic Acid Bacteria (LAB) which are described to play an important role on artisanal cheese properties (Brazil, 2013; Martins et al., 2015; Kamimura et al., 2019). These native or wild cultures exhibit interesting characteristics, since they are thought to survive adverse environmental conditions and predominate in this ecosystem by the production of antimicrobial substances (like bacteriocins, organic acids, ethanol, CO_2, hydrogen peroxide, diacetyl, fatty acids, etc.), resisting in competition with other microorganisms and promoting microbial stability of the final product. For these reason LAB has been used in food preservation for decades, presenting a generally recognized as safe (GRAS) status.

LAB is widespread in nature and often dominate the microflora of milk, milk products and fermented foods in general. They can be used as commercial starter cultures due their various metabolic characteristics, contributing to the flavor, texture and commonly to the nutritional features of dairy products (Ayad et al., 2004). In addition to these technological properties, LAB may play a functional role, due bioactive peptides synthesis resulting from proteolytic activity in the milk fermentation process exerting several functions, i. e., hypotensive (through the inhibition of ACE-angiotensin-I-converting enzyme) and probiotic properties (Nielsen et al., 2009).

Thus, characterisation and selection of wild LAB from cheese and other artisanal milk-based products made around the world has been considered a promising strategy to search for new industrially important cultures to explore and apply the natural biodiversity for new interesting properties (Scatassa et al., 2015; Carafa et al., 2016; Fguiri et al., 2016; Wang et al., 2016). In this work we started out with Brazilian artisanal cheeses and searched for wild LAB that originated from the endogenous cheese microbiota and which could possibly be used to manufacture dairy products under aseptic conditions but

with regional features. It was also important to investigate the influences of isolation source and some technological properties, aiming to corroborate the “Protected designation of origin (PDO)” status. In order to meet the demand of consumers for safer foods and showing functional effects, long shelf life and clean label manufacturing was part of the objectives of the present study. For this we evaluated the technological and bio-preservation characteristics of 1,002 lactic bacteria isolated from 11 types of Brazilian artisanal cheeses from different regions and to access their potential for application in the dairy industry as starter and/or adjunct cultures.

2. Materials and Methods

2.1 Cheese sampling, LAB counting and isolation

Five hundred and seventy eight (n=578) Brazilian artisanal cheeses of eleven different types were analyzed, being: Araxá, Cerrado, Serra da Canastra, Serro and Campo das Vertentes, from Southeast (n = 262), Coalho and Manteiga, from Northeast (n = 101), Serrano and Colonial, from South (n = 99), Caipira from Midwest (n = 108) and Marajó, from North (n=8). These cheeses weighed approximately 1 kg and were bought in different households or small grocery dairies, between July/2014 and February/2015. LAB count was performed according to Dowes & Ito (2001), using the pour plate method in two standard media: De Man agar, Rogosa and Sharpe - MRS (Merck, Darmstadt, Germany), specific for *Lactobacillus* sp. and M17 agar (Sigma-Aldrich, St. Louis, USA), specific for *Streptococcos* and *Lactococcos*. Paltes were covered with 10 mL of 1.2% bacteriological agar to create a microaerophilic condition (Kasvi, Curitiba, Brazil) and incubated at 30 °C for 72 hours. The count was expressed as log CFU/g. Both media were supplemented with natamycin (50 mg/mL) (Danisco, Grindsted, Denmark) to avoid fungal

contamination. Five characteristic single colonies from MRS and M17 of each cheese were selected for purification and incubated at 30°C for 48 h again in the respective media. Gram (Gregersen, 1978) and catalase (3 % hydrogen peroxyde) tests as well as morphologic observation, were conducted in order to eliminate non-LAB isolates (e.g. Gram-positive, catalase-negative and cocci or rod morphology). Cultures identified as LAB were kept in MRS broth (Acumedia, Neogen Corporation, Lansing/MI) with 30% glycerol at -80°C. A total of 1,002 isolates were chosen for further analysis.

2.2 Technological potential

The initial evaluation of the technological potential of 1,002 lactic acid bacteria was carried out by means of acidification profiles, proteolytic and lipolytic activities, and NaCl, pH and bile salts tolerance. The antimicrobial activity were also tested against the pathogens: *Listeria monocytogenes* ATCC 35152, *Staphylococcus aureus* ATCC 19095 and *Escherichia coli* ATCC 25922, considered as standards; *Staphylococcus saprophyticus* NIZO 01392, *Staphylococcus aureus* NIZO 04185 and *Listeria monocytogenes* NIZO 00397, isolated from dairy factories; and also, a representative species used as starter culture: *Lactococcus lactis* NCDO712.

2.2.1 Acidifying, proteolytic and lipolytic assays

Acidifying, proteolytic and lipolytic capacity were assayed according to the methodologies of Bachmann et al. (2013) and Sahraoui et al. (2015), with modifications. For the acidification curves, cells were pre-cultured overnight in MRS broth at 30 ºC and inoculated (~10^7 CFU/mL) in 200 uL of milk (2%) containing 1 uM of -5-(6)-carboxyfluorescein (#21877, Sigma-Aldrich) in multiple wells of a black microplate with

transparent bottom. Fluorescence emission at 520 nm (Excitation 485 nm) was measured in regular intervals of 1.5 hours in a microplate reader (Fluorostar, BMG LABTECH, Ortenberg, Germany). A calibration curve was constructed with milk at pH 4, 4.5, 5.0, 5.5, 6.0 and 6.5 containing the same amount of the pH indicator and under the same conditions. The acidification rates were measured based on the decreasing fluorescence intensity of the pH-dependent indicator carboxy-fluoresceine and all the data analysed using the microplate package in software R.

For the determination of proteolytic activity, 2 µL (~10^8 CFU/mL) microdroplets of cultures grown overnight in MRS broth were spotted onto the surface of reconstituted skimmed milk agar (RSMA) medium containing 10% of milk (m/v) and 2% of agar (m/v). Proteolytic positive strains were identified upon the formation of clear zones around the cells. For the determination of lipolytic activity, 2 µl micro-drops of the cultures grown overnight in MRS broth were poured onto plates containing lipolytic medium (meat peptone: 2.5 g / L; casein peptone: 2.5 g / L; yeast extract: 3 g / L; Agar-agar: 12g / L), added with 1% tributyrin and 0.0025% (w/v) red phenol as a coloured indicator for the production of butyric acid. The appearance of yellow halos around the cells indicated positive activity. The results were expressed in high (+++), medium (++) and low (+) activity, according to halo size or intensity. Figures 1 and 2 show the protocols used for high throughput screening of OD stress curves and acidification curves, adapted for 96-well microplates.

[insert Figure 1 here]

[insert Figure 2 here]

2.2.2 Bacteriocin activity against pathogenic and spoilage bacteria

The LAB strains were tested for their ability to produce bacteriocins against pathogens previously described by Charteris et al (1998), with some modifications. Each pathogen was cultured in BHI – Brain heart infusion - broth and cell suspensions (10^9 CFU/mL) were prepared according to Sant'Ana et al. (2012). Ten microliters of frozen stock of each pathogen were inoculated in 10 mL of Brain-Heart Infusion Broth (brand) and incubated at 37 ºC for 24 h. The cell culture was centrifuged (4000 g; 10 min) and the pellets were washed twice with Phosphate Buffer Solution (pH 7.2). The OD of cell suspensions was adjusted to approximately 1 at 600 nm (≈ 10^9 CFU/mL). Fifty microliters of each pathogen suspension were diluted in 50 mL of BHI agar and poured into petri dishes of 12 cm of diameter until solidification. Then, 3 uL of cell-free supernatant, obtained by filtering using a 96-microplate filter (HTS 96-Well Filter Plates with MCE Membrane, Merck, Darmstadt, Germany) of each LAB were spotted onto the top of each plate in order to assess their bacteriocin activity against the pathogens, indicated by a clear zone around the spot. One plate containing just MRS medium added with purple bromocresol (80 mg/L) were also inoculated to ensure that the pH would not interfere in the inhibition. Figure 3 shows the protocol used for high throughput screening of antimicrobial assessment, adapted for 96-well microplates.

[insert Figure 3 here]

2.2.3 Growth at different stress levels via $OD600_{nm}$

To evaluate strain resistance to stress conditions and select the most robust strains, overnight (30 °C, 16–18 h) cultures were inoculated (2% v/v) in microplates

containing modified MRS broth with different concentrations of NaCl (4, 6 and 8%), bile salts (0.5 and 1%) and different pH (3, 4 and 8). Unmodified MRS broth was used as control. After inoculation growth was followed through OD_{600nm} measurements at 30 ºC, every 2 h in a Plate Butler® Robotic Systems. The data were analysed using a microplate package to calculate the maximum growth rates and OD values of each strain using the software R version 3. 5. 1.

2.3 Identification of selected strains

2.3.1 MALDI-TOF MS - Matrix Assisted Laser Desorption/Ionisation-Time of Flight Mass Spectrometry

Representative strains of LAB isolated from artisanal cheeses samples were submitted to MALDI-TOF identification by a Dutch hospital called Ziekenhuis Gelderse Vallei. The bacterial suspensions for MALDI-TOF analysis were prepared as follows: a single LAB colony grown on MRS agar were diluted in 300 µL of DNA free sterile water until a turbidity sample was obtained. Then, 900 µL of absolute ethanol were added and the whole sample was vigorously mixed and sent to the hospital for further identification.

The suspension was centrifuged (13.000 x g; 2 min), the supernatant was removed and the pellets were dried at room temperature until dry or at least for 5 min. The bacterial pellet was resuspended in 20– 50 µL of formic acid (70 % in water) and the same amount of acetonitrile.

After centrifugation (2 min at 13.000 x g), 1 µL of the supernatant was spotted onto a sample position of a polished steel MALDI target plate and dried at room temperature. Subsequently, 1 µl MALDI matrix (solution of α-cyano-4-hydroxycinnamic acid (HCCA) in 50 % acetonitrile/2.5 % trifluoro-acetic acid) was added to the spot and

dried again (30 min). The MALDI target plate was introduced into the MALDI-TOF mass spectrometer for automated measurement and data interpretation. MALDITOF profile mass spectra were imported into the MALDI Biotyper 3.0 software and processed automatically after measurement. The logarithm of the score (log[score]) was displayed as the matching result. The MALDI Biotyper output was a log(score) between 0 and 3.0, which was calculated from a comparison of the peak list from an unknown isolate with the reference MSP in the database. A log(score) ≥1.7 indicated identification at the genus level, while a log(score) ≥2.0 was set as the threshold for a match at the species level.

2.4 Statistical Analysis

The data of technological potential was submitted to multivariate analysis of principal components using the software *Pirouette version 3.11*. Correspondence analysis was performed using the software *PAST version 3.26*. Analysis of statistical significance was performed by ANOVA. Student's t-test was used, when necessary, to discriminate differences between means. Differences with P-value < 0.05 were considered statistically significant.

3. Results and Discussion

This study aimed to assess the applicability of 1,002 wild bacterial strains isolated from Brazilian artisanal cheeses for their potential in dairy fermentations. The conditions tested include the ability to resist some stress conditions generally present in the cheese manufacturing process (low pH, lack of nutrients, high osmotic pressure), the potential to acidify milk as starter or adjunct cultures and the ability to inhibit spoilage

organisms. Figures 4 and 5 exemplifies how data were interpreted for proteolytic, lipolytic and antimicrobial analysis, being qualitatively classified as: weak (+), moderate (++) and strong (+++) activity. To analyse the data from OD stress curves tests, maximum OD (maxOD) reached by each strain was divided into resistant (OD > 0.8), moderate (0.39 < OD <0.8) and weak (OD < 0.39), when compared to the control (just MRS broth), where all cell reaches an OD > 0.8. Figure 7 summarizes the results of all tests to assess technological potential (production of extracellular enzymes) and probiotic (resistance to bile salts, low pH and ability to inhibit the growth of pathogenic microorganisms) of all strains, according to its isolation source (cheese type). The ability to release proteolytic and lipolytic enzymes and to acidify milk are related to some organoleptic features in cheese, like aroma and texture.

[insert Figure 4 here]

[insert Figure 5 here]

The acidification rates from each population of isolates present in different types of cheeses are shown in the box plot represented by figure 6. There was a great diversity in LAB population within cheeses, mainly in Araxá, Campo das Vertentes, Caipira, Coalho and Serro. These values ranged from 0 to 0.16 ($\Delta pH.h^{-1}$) and were considered low when compared to the control (~ 0.7 $\Delta pH.h^{-1}$) strain of *Lc. Lactis* NCD 0712, a strain isolated from a dairy starter culture. For this reason, the variable acidification rate did not join the PCA. The strains with higher acidification rates were identified as *Lactobacillus paracasei* and *Lactobacillus brevis* by MALDI-TOF.

[insert Figure 6 here]

Figure 5 shows the halos formed in growth inhibition tests against *L. monocytogenes* ATCC 35152 using the supernatant of LAB cultures. Nine isolates were able to inhibit the growth of this pathogen. None of the LAB tested showed growth inhibitory activity against starter cultures of *Lactococcus lactis* NCD 0712 or *Lactococcus lactis* KF 147. These data disagree with antagonistic tests performed with the same strains by Campagnollo et al. (2018) which showed inhibitory effect against species of dairy isolated species of *Listeria monocytogenes* and *Staphylococcus aureus* in antagonism tests (co-culture in agar plates). This is probably due the mechanisms involved in bacteriocin formation, which generally require cell contact with target bacteria through induction factors. The protocol of this study applied just the sterile supernatant in order to ensure an inhibition by extracellular bacteriocin-like substances. MALDI-TOF analysis of the growth inhibiting strains were identified as *Lactobacillus plantarum* and *Lactobacillus buchneri* species.

Based on the data shown in figure 7, all strains were submitted to perform a PCA (Figure 8). The results showed 7 groups of isolates (Figure 8.A) that relate to the studied variables (Figure 8.B).

[insert Figure 7 here]

[insert Figure 8 here]

The features of each group resulted from PCA analysis are summarized in Figure 9. All isolates belonging to groups 1 and 2 were characterized by lipolytic activity. Group 2 was distinguished by the presence of strains able to produce both lipolytic and proteolytic extracellular enzymes as well as by resisting salt concentration of 4% (w/v). However, only a small percentage of these strains had resistance to low pH and to the presence of bile salts. According to MALDI-TOF analysis, they were identified as *Pediococcus acidilactici*, all isolated from Marajó cheeses, being considered as adjunct cultures that act on the aroma formation of the final product. It is interested to see that the Marajó cheeses show high fatty acids contents (Lourenço et al., 2005) which might favour lipolytic organisms. In group 4, all strains showed proteolytic activity.

[insert Figure 9 here]

Groups 5 and 6 were distinguished by the presence of isolates capable of inhibiting *L. monocytogenes*, as well as by having higher resistance to salt at 4 and 6% (w/v). In group 7, the isolate 3_QB_M17_199 also exhibited these characteristics. All these strains were isolated from Minas Artesanal cheeses from Araxá, Campo das Vertentes and Canastra microregions and were identified as *Lb. plantarum*. In group 5, however, the strain *Lactobacillus bucherie* isolated from Serrano cheese also showed proteolytic activity. Based on these properties these isolates are good candidates to be applied as biopreservatives in the production of artisanal cheese. In one study using *Lb. plantarum* strains, they also found a connection between the strains and their source of isolation. As an example, strains isolated from Oscypek cheese showed stronger activity

against *L. monocytogenes*, whereas strains isolated from korycinski cheese were more active against *E. coli* (Oldak et al, 2017).

Figure 10 shows the distribution of acidification, proteolytic, lipolytic and bacteriocin activity as a function of the region and, therefore, the type of cheese from which the strains were isolated. The isolates from Minas Artesanal cheeses stand out for presenting better performance in all tests evaluated. Some isolates from Coalho cheese stood out due to their proteolytic activity, which might present a greater potential for their application as adjunct cultures, acting on ripening of the cheeses. These strains can be classified as Nonstarter lactic acid bacteria – *NSLAB*.

[insert Figure 10 here]

Although the role of *NSLAB* is still relatively unclear, some reports demonstrated that *NSLAB* possess wide range of peptide hydrolytic enzymes, which causes an increase of the number of short peptides and free amino acids. In addition, *NSLAB* possess higher lipase and esterase activities compare to SLAB, leading to higher production of free fatty acids. The high contribution of *NSLAB* during ripening is strengthened by the fact that NSLAB could survive the harsh condition of cheese ripening (low pH, low water activity and high salt content), while the amount of SLAB decrease significantly (Guidone et al., 2014).

Representative strains from each group were characterized using MALDI-TOF analysis which resulted in the following species identifications: *Lactobacillus brevis*, *Lactobacillus bucherie*, *Lactobacillus curvatus*, *Lactobacillus fermentum*, *Lactobacillus paracasei*, *Lactobacillus paraplantarum*, *Lactobacillus plantarum*, *Lactobacillus*

rhamnosus, *Lactococcus garviae*, *Lactococcus lactis*, *Lactococcus* spp., *Leuconostoc mesenteroides*, *Pediococcus acidilactici* and *Pediococcus pentosaceus*. Many of these species are current applied as probiotics in foods and the animal feed industry. *Lactobacillus* genus is also widely used as starter to manufacture a broad variety of fermented products, such as cheese, yoghurt, vegetables, etc. The extended use of *Lactobacillus* species is mainly related to their GRAS (Generally Recognized as Safe) status. With this study we show how traditional artisanal cheeses from Brazil may constitute a good reservoir for new adjunct and biopreservatives LAB spp.

The figures 11, 12 and 13 show growth curve characteristics (μmax and maximum OD) per species in response to different concentrations of NaCl, Bile salts and low pH values. It is noticeable that *Lb. plantarum* strains performed well in all conditions, followed by *Lb. brevis*, isolated from Minas Artisanal cheeses from Araxa and Campo das Vertentes microregions, respectively. Parente et al. (2010) reported that 60 out of 63 *Lb. plantarum* strains remained viable after exposure to 17.5% (w/v) NaCl for 1 h. The osmotic resistance of this species is particularly known, and due this attribute they can ferment food with high salt concentration (0.5 - 10 %) and survive better to lyophilization and spray dried process applied in the production of marketable ferments.

[insert Figure 11 here]

[insert Figure 12here]

[insert Figure 13 here]

We also found a positive correlation ($p = 0.36$) between the growth rates obtained from pH at 4 and NaCl at 4% (w/v) experiments. This was expected since pH decreases during LAB culture growth and general stress proteins, such as DnaK, DnaJ, GroES, GroEL chaperons, are synthesized. These proteins are also involved in heat and osmotic resistance (Carvalho et al., 2004).

Survival and technological performance of strains used as starters, adjuncts and/or probiotics after subjecting them to adverse conditions (such as bile, pH, lack of nutrients, high osmotic pressure, etc.), either during storage or fermentation, are essential for a successful industrial application. To be classified as probiotics strains need to resist gastric acidity and bile salts, according to FAO/WHO guidelines (2002), among other analyses. In this sense using the “robustness” of strains against stress factors as selection criteria allows for well-educated choices. To increase the applicability of individual strains they can be adapted to various stress conditions. *Lactobacillus* and *Bifidobacterium* genera are the most commonly used as potential probiotics and they are included in functional fermented food, but other species, like *Lb. plantarum* have emerged as potential probiotics able to also act as starter, an advantage over most probiotic species currently used (Ferrando et al., 2016).

Our results demonstrate the relevance of screening cell technological resistance when selecting cultures with probiotic and technological potential, since different stress factors usually applied during food processing might considerably affect viability or/and performance of the strains.

4. Conclusion

The highlight of this study was to set a multivariate high-throughput screening in order to obtain insights related to the favourable combination of functional and technological properties of LAB and to select them for further application as starters in fermented food. *Lb. plantarum* was highly isolated from Brazilian Artisanal Cheeses and showed a large intraspecific heterogeneity. The type of cheese influenced some properties analysed, such as the production of bacteriogenic compounds bacteriocins, produced by *Lb. plantarum* and *Lb. boucherie* from Minas Artisanal Cheeses (from Southeast region); the production of lipolytic enzymes by *Pediococcus acidilactis* from Marajó cheese (from North region). In general, the strains presented a good potential to be applied as biopreservatives, probiotics and as adjuncts cultures, contributing to flavor and texture during ripening.

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FIGURES

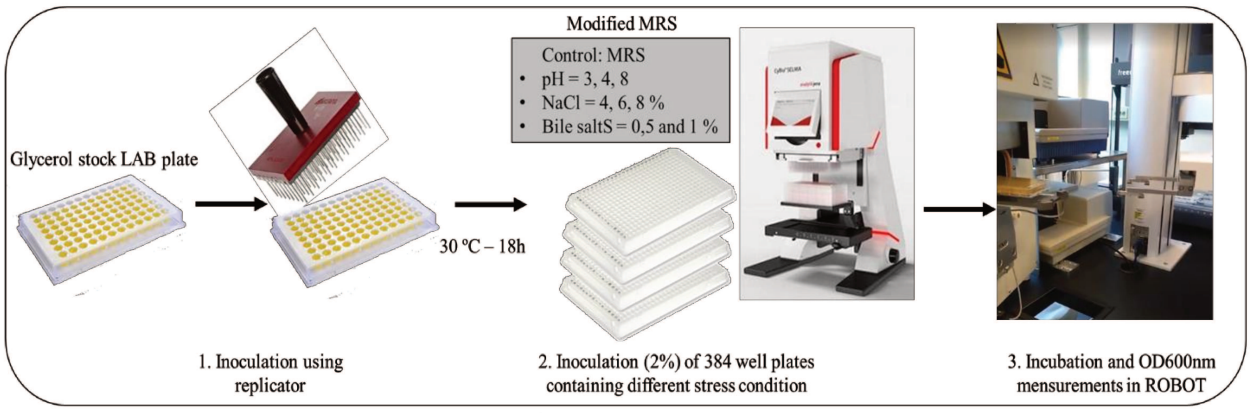

Figure 1. Detailed protocol for OD stress curves.

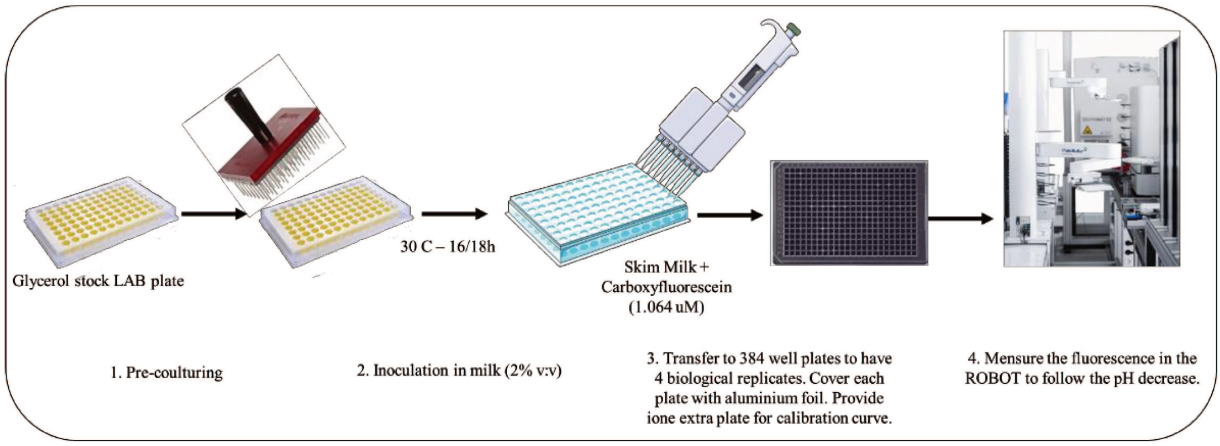

Figure 2. Detailed protocol for Acidification curves.

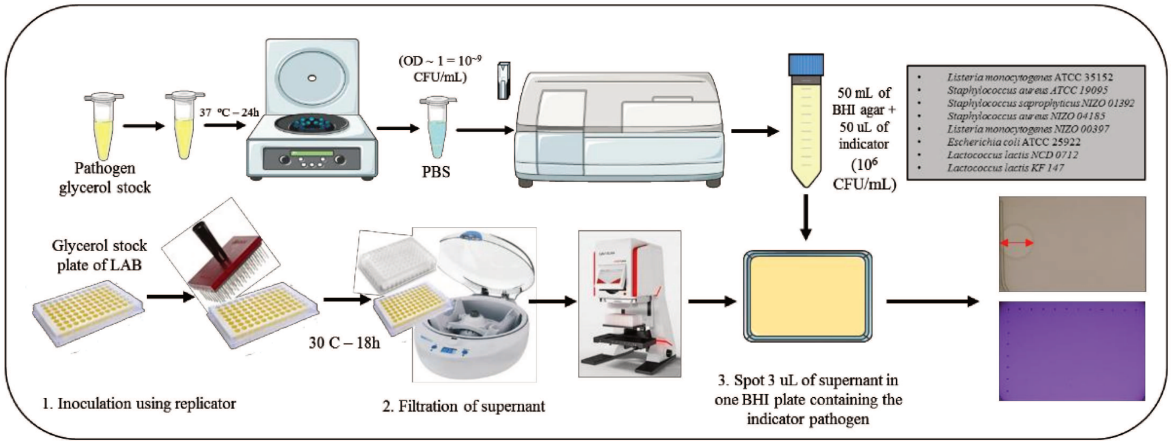

Figure 3. Detailed protocol for bacteriocin test.

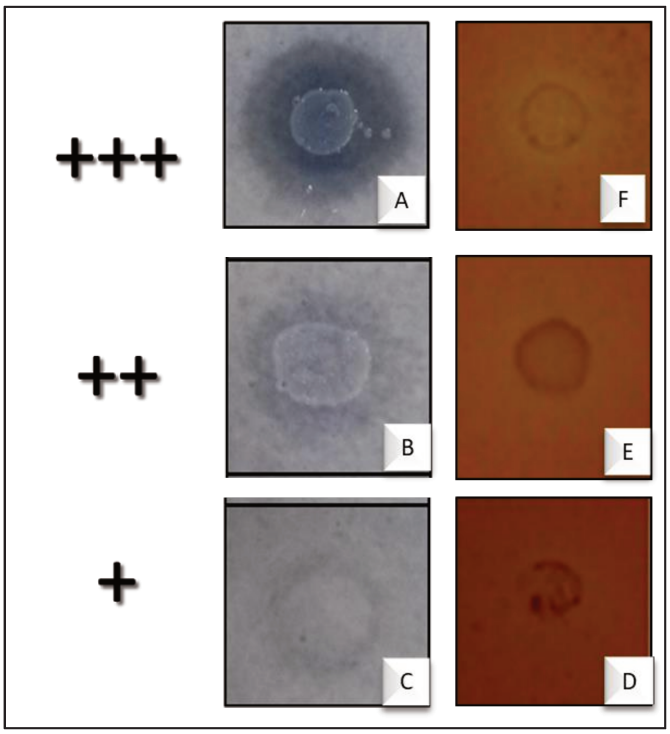

Figure 4. Plates resulted from proteolytic and lipolytic extracellular enzymes screening. The strains were classified in strong (A), moderate (B) and weak (C) proteolytic according to the halo formed surrounding the bacteria. For the lipolytic activity, the strains were classified as strong (F), moderate (E) and weak (D) producers, according to the intensity of yellow halos formed around the spot of each strain onto the plate, indicating the production of butyric acid.

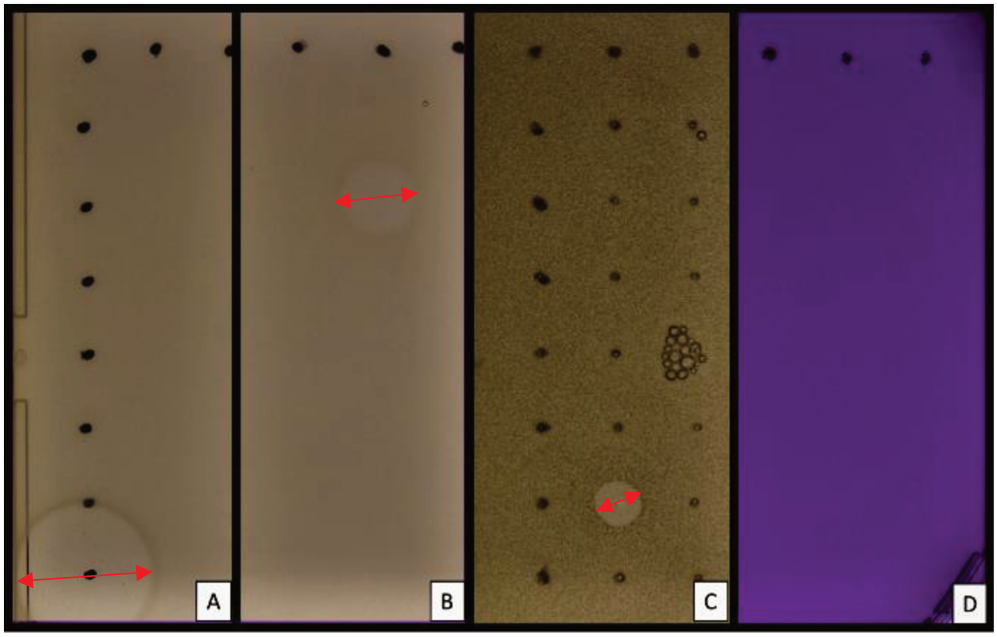

Figure 5. Bacteriocigenic activity determined based on to the identified halo size, being classified as high (A), moderate (B) or weak (C) producers; Control plate (D) containg just the supernatans of the LAB without the pathogen. The pathogen on these plates are: *Listeria monocytogenes* ATCC 32152 (A and B) and *Staphylococcus aureus* ATCC 19095 (C).

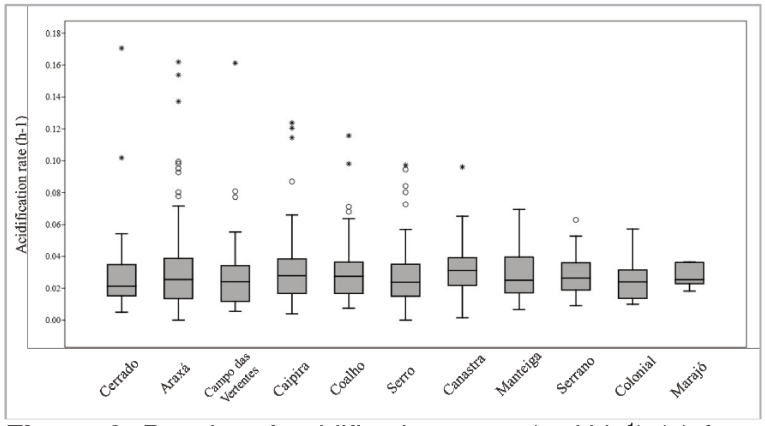

Figure 6. Boxplot of acidification rates (ΔpH.h^{-1}) (y) from LAB isolated from different cheese sources (x).

	Prot+++	Prot++	Lip+++	Lip++	4.NaCl_++	4.NaCl_+++	6.NaCl_++	6.NaCl_+++	8.NaCl_++	8.NaCl_+++	Bac+_Lm	0.5BS_++	0.5BS_+++	1.0BS_++	1.0BS_+++	pH3_+	pH4_++	pH4_+++	pH8_++	pH8_+++	
C. das Vertentes (n=90)	2,2	10,0	0,0	0,0	34,4	52,2	27,8	30,0	10,0	7,8	2,2	22,2	35,6	4,4	20,0	41,1	31,1	28,9	1,1	35,6	Southeast
Cerrado (n=64)	1,6	3,1	0,0	1,6	34,4	45,3	29,7	25,0	3,1	0,0	0,0	23,4	10,9	1,6	1,6	7,8	21,9	6,3	0,0	6,3	
Araxa (n=167)	0,6	14,4	1,2	0,0	43,7	39,5	47,9	17,4	0,0	0,0	0,0	23,4	26,3	0,0	0,6	9,6	39,5	23,4	0,6	7,8	
Canastra (n=108)	0,0	13,0	0,9	0,0	35,2	44,4	25,9	32,4	9,3	1,9	13,0	16,7	19,4	5,6	9,3	22,2	32,4	15,7	0,0	20,4	
Serro (n=115)	0,0	29,6	0,0	0,0	38,3	43,5	25,2	25,2	5,2	0,0	0,0	20,0	18,3	0,0	7,8	21,7	33,0	13,0	0,9	19,1	
Caipira (n=167)	0,0	14,4	0,0	1,2	36,5	42,5	29,9	28,7	6,6	1,8	0,0	21,0	20,4	0,6	9,0	27,5	28,1	20,4	0,0	21,0	Center-West
Coalho (n=122)	0,0	12,3	0,0	0,0	20,5	50,0	21,3	35,2	15,6	3,3	0,8	9,8	33,6	7,4	18,0	45,9	25,4	15,6	0,8	38,5	Northeast
Manteiga (n=28)	0,0	10,7	0,0	0,0	17,9	50,0	17,9	35,7	3,6	3,6	0,0	14,3	28,6	3,6	7,1	32,1	10,7	17,9	0,0	28,6	
Colonial (n=68)	0,0	5,9	1,5	2,9	33,8	32,4	25,0	17,6	2,9	0,0	0,0	23,5	20,6	0,0	4,4	23,5	44,1	10,3	0,0	14,7	South
Serrano (n=61)	0,0	14,8	1,6	0,0	42,6	41,0	47,5	26,2	6,6	1,6	1,6	21,3	19,7	0,0	8,2	14,8	41,0	27,9	0,0	13,1	
Marajó (n=12)	0,0	58,3	0,0	41,7	50,0	25,0	66,7	16,7	0,0	0,0	0,0	8,3	0,0	0,0	0,0	0,0	33,3	0,0	0,0	0,0	North

Figure 7. Percentage of LAB strains, per cheese type, and their pro-technological potential. Namely, from the left to right: ability to produce proteolytic (Prot) and lipolytic (Lip) enzymes, in high (+++), moderate (++) and low (+) amounts; to resist NaCl stress at 4, 6 and 8 % (w/v); to produce bacteriogenic activity against *Listeria monocytogenes* ATCC 35152 (Bac+_Lm); to resist Bile salts stress at 0.5 and 1.0 % (w/v) and different pH values (3, 4 and 8).

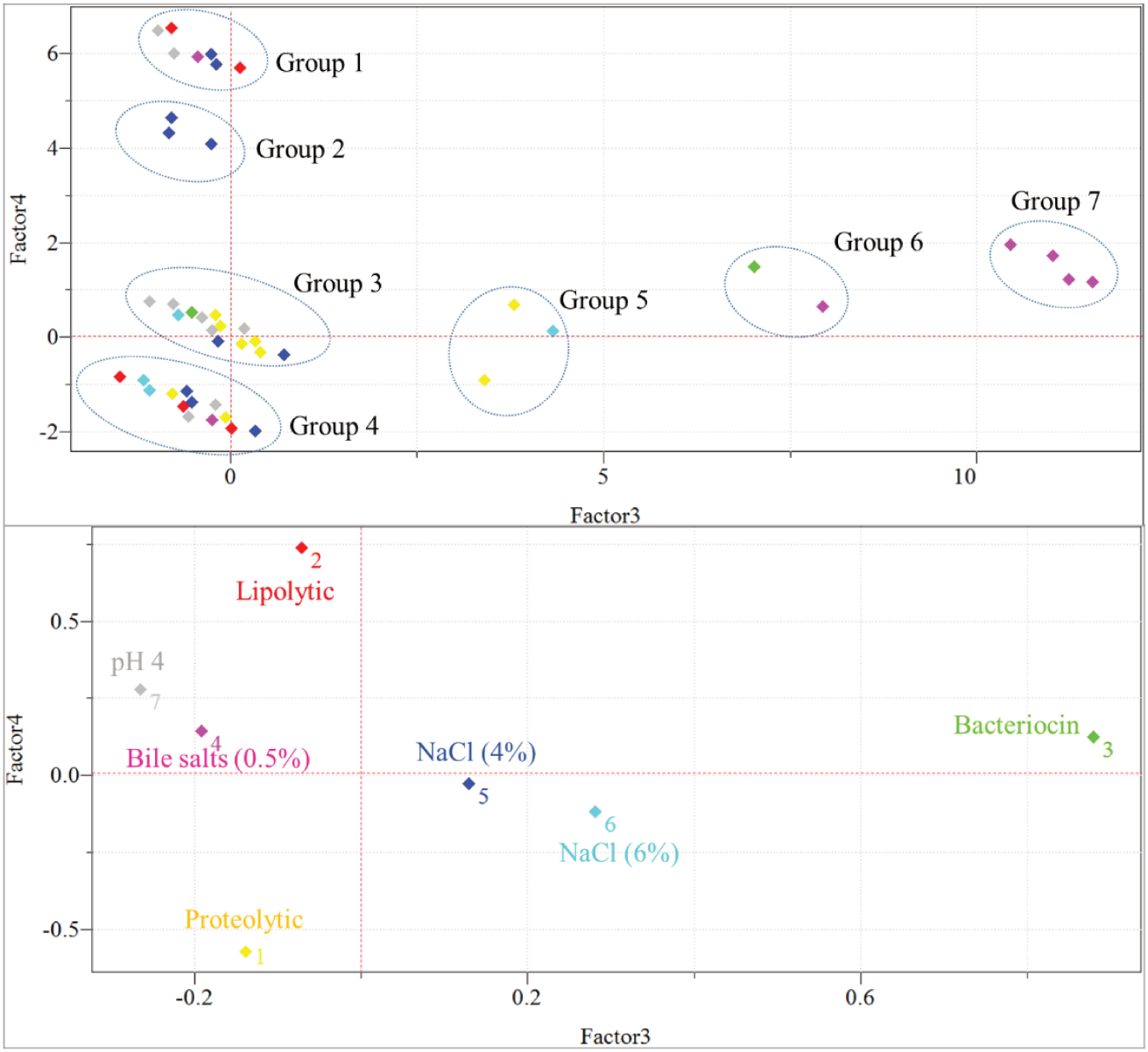

Figure 8. Principal component analysis (PCA) score plot of LAB strains (A) isolated from Brazilian Artisanal Cheeses using the factors 3 and 4 (PC3 vs PC4) (B) obtained from the PCA made in *Pirouette* vs. 3.11. The strains are collored by origin of each cheese. Factors used: High and moderate lipolitic (Lipolytic), proteolytic (Proteolytic) strains; High and moderate resistant strains to low pH (p H4), Bile salts at 0.5 % and NaCl at 4 and 6% (w/v); Bacteriocin activivity against *Listeria monocytogenes* ATTC 35152.

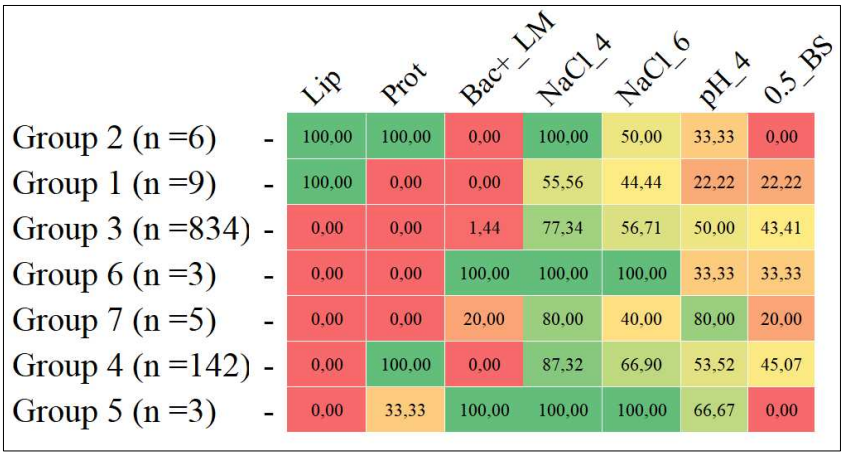

Figure 9. Resulting groups from PCA analysis of LAB strains, according to their phenotypic features. Factors used: High and moderate lipolitic (Lipolytic), proteolytic (Proteolytic) strains; High and moderate resistant strains to low pH (p H4), Bile salts at 0.5 % and NaCl at 4 and 6% (w/v); Bacteriocin activivity against *Listeria monocytogenes* ATTC 32152. "n" = number of isolates per cheese group.

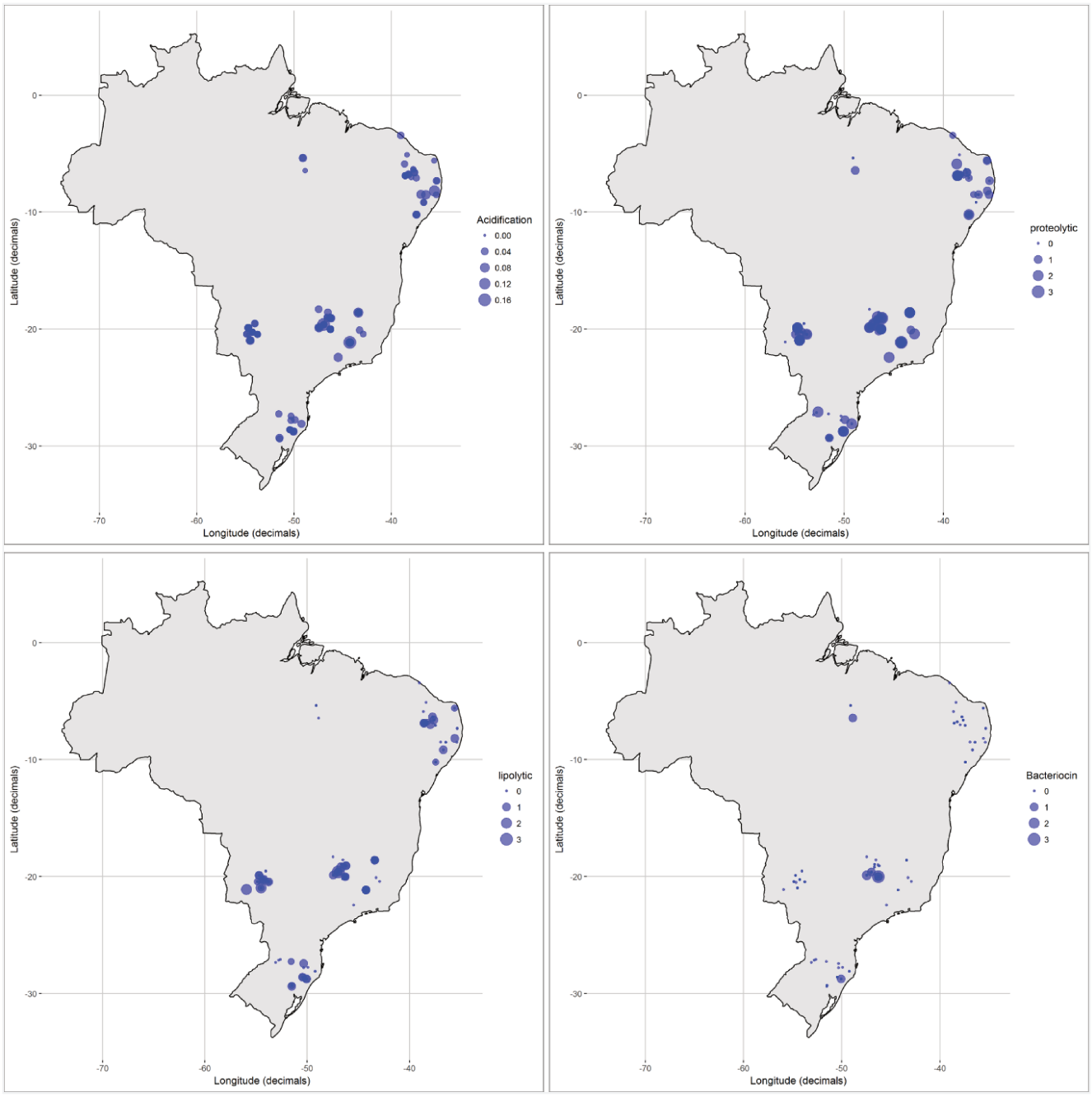

Figure 10. Describes the results of technological analysis: rate of acidification (A), proteolysis (B), lipolysis (C) and bacteriocin activity from LAB strains isolated from artisanal cheeses made in different Regions of Brazil. Circle points are colored according to intensity indicated by the numbers 0, 1, 2, 3: null, low, moderate and strong activity, respectively.

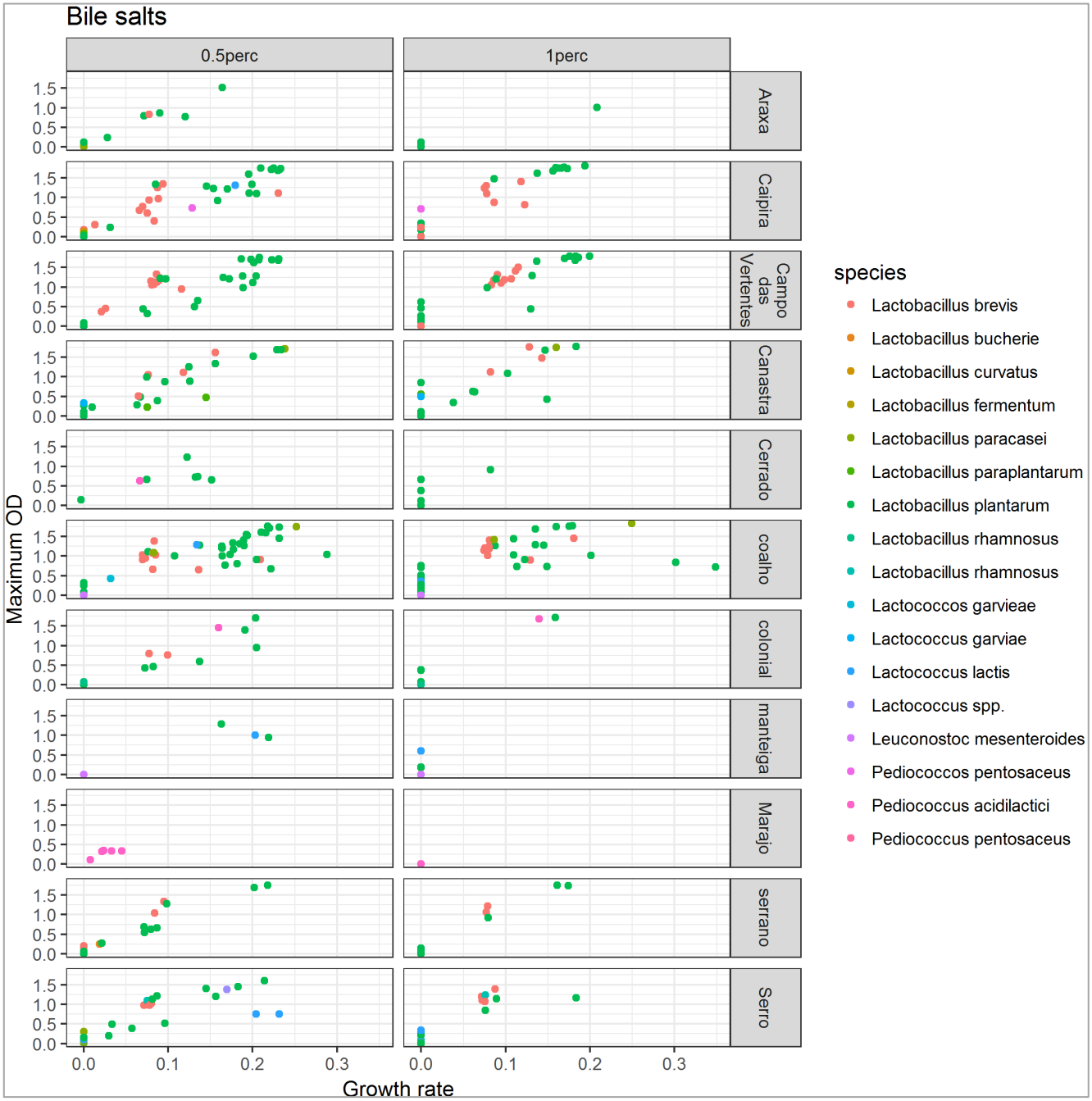

Figure 11. Bile salt resistance of different species of LAB (expressed as maximum optical density and growth rate) isolated from Brazilian artisanal cheeses (cheeses given in vertical panels). Bile salts were added at 0.5 and 1.0 % (w/v) (horizontal panels) to MRS broth. Without bile salts, all strains reached OD > 0.8.

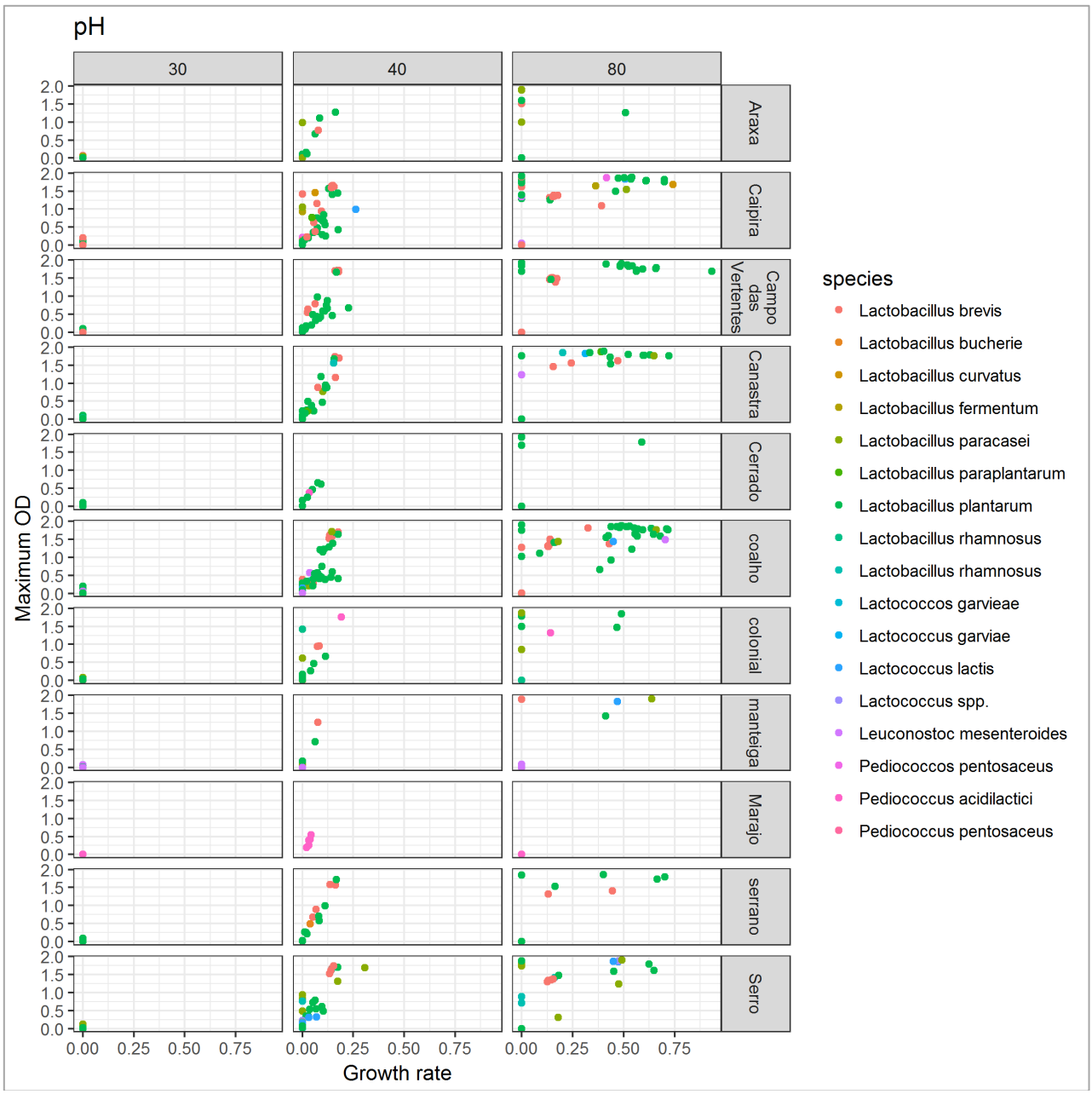

Figure 12. Resistance of different species of LAB (expressed as maximum optical density and growth rate) isolated from Brazilian artisanal cheeses (cheeses given in vertical panels) to pH 3, 4 and 8 in modified MRS broth (horizontal panels). Without bile salts, all strains reached OD > 0.8.

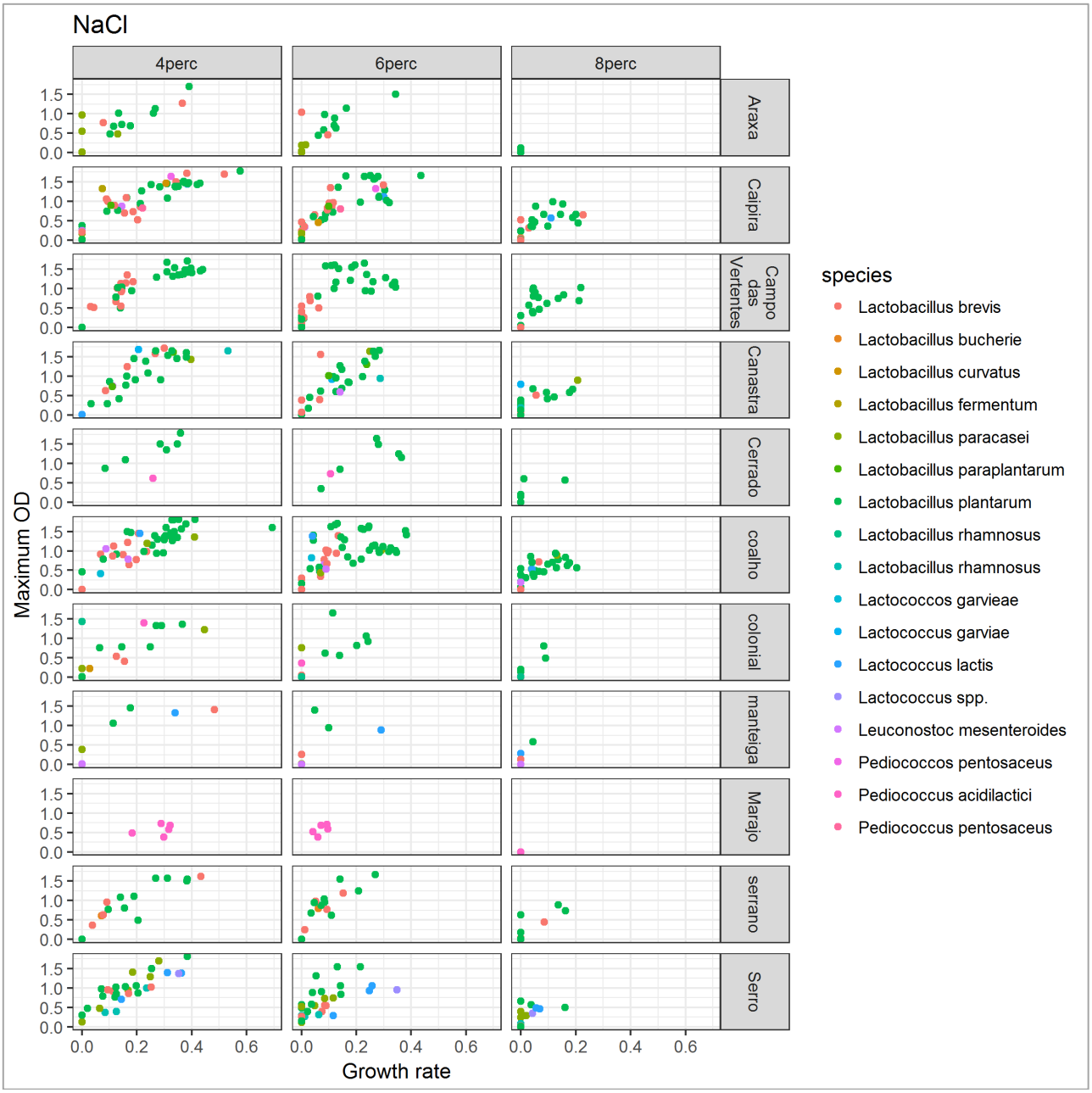

Figure 13. Resistence of different species of LAB isolated from Brazilian artisanal cheeses to NaCL at 4, 6 and 8% (w/v) in MRS broth.

CAPÍTULO 2: Brazilian artisanal cheeses as a potential source of wild lactic acid bacteria to be applied in dairy industry. Part II: Assessment of *Enterococci* sp. occurrence and their technological and safety features

Artigo formatado de acordo com as normas de submissão da revista "Food control"

Brazilian artisanal cheeses as a potential source of wild lactic acid bacteria to be applied in dairy industry. Part II: Assessment of *Enterococci* sp. occurrence and their technological features

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Abstract

Although *Enterococci* sp. is naturally found in many fermented foods, some issues regarding its safety has emerged since a high number of Vancomycin-resistant-*Enterococci* (VRE) has been arising, constituting a risk for consumers. Thus, this study aims to provide information about the occurrence of *Enterococci* sp. strains in traditional Brazilian cheese samples collected from the five main regions, focusing on their prevalence, technological (proteolytic, lipolytic and bacteriogenic potential) and safety (haemolysin activity and growth at commercial VRE selecting mediums) aspects. A total of 1,512 lactic acid bacteria were isolated of which 576 (38.09 %) were identified as *Enterococcus sp.* by 16sRNA sequencing. *E. faecium* (58.73%) was identified as the dominant species, followed by *Enterococcus faecalis* (31.75 %), *Enterococcus durans* (4.76 %), *Enterococcus hermanniensis* (1.59 %) and *Enterococcus gilvus* (1.59%). All data were analysed by multivariate statistics, and our study highlighted the presence of E*nterococci* as a naturally component of endogenous microbiota of Brazilian artisanal cheeses, mostly in Marajó, Coalho and Manteiga, possibly due to the curd cooking step during the process. It was possible to identify *Enterococci* (63.19%) with potential for application as starter cultures and/or biopreservatives in the production of fermented products derived from milk due to the absence of beta-haemolysin production and resistance to vancomycin. Strains from Serrano and Cerrado cheeses stands out due their bacteriocin (enterocin) inhibition against *Listeria monocytogenes* ATCC 35152, which is a very useful criterion when screening strains with probiotic potential. In summary, multivariate statistics combined with high throughput tools enabled to open new possibilities for *Enterococci* as starters.

Keywords: Brazilian artisanal cheese, lactic acid bacteria, *Enterococci*, adjunct cultures, biotechnology, food quality, food safety, vancomycin resistance, endogenous microbiota.

1. Introduction

Artisanal food products have been the subject of intense research over the decades due to the presence of autochthonous microorganisms that play a fundamental role in the development of their distinguished flavor and aroma. These include artisanal cheeses, whose microbiota are highly associated with their region of origin, the use of raw milk in their processing as well as empirical and traditional small-scale production techniques, passed from generation to generation for more than 200 years. In Brazil, this kind of cheeses show a great appeal to local consumers and have a great diversity of lactic bacteria present, such as strains of the following genera: *Lactobacillus*, *Lactococcus*, *Leuconostoc, Aerococcus*, *Weissella*, *Pediococcus*, *Carnobacterium*, *Vagococcus*, *Brochotrix*, *Atopobium*, *Tetragenococcus* and *Enterococcus* (Kleerebezem, Kuipers and Smid, 2017).

Recent studies reporting the application of these endogenous lactic acid bacteria as starter in dairy foods have raised doubts about the use of strains belonging to the genus *Enterococci* since they have been related as frequent cause of hospital infections (such as endocarditis, bacteremias, and urinary tract infections) due to an increased resistance to certain antibiotics, especially vancomycin and ampicillin (Lebreton, Willems and Gilmore, 2014). From 1988, there was an increase in the number of vancomycin-resistant *Enterococci* (VRE) in Europe and around the world, which represents a big risk to immunosuppressed patients. In this scenario, *E. faecalis* and *E. faecium* strains represent 80 and 20% of clinical cases, respectively, with increasing numbers (Cavicchioli et al., 2017; Sanlibaba and Senturk, 2018). In a study done in Brazil,

strains of *E. faecalis* species were also considered as causative agents in dental infections (Andrade and Pinto, 2011).

Despite the gastrointestinal tract of warm-blooded animals being considered as a natural habitat, the genus *Enterococci* is widely dispersed in nature (soil, plants, surface waters, etc.), due to its adaptability to diverse environments, such as large ranges of temperature, pH, and salt concentrations. Such phenotypic behaviour as a generalist allows it to frequently occur in foods of animal origin such as meats and cheeses, which are rich in carbohydrates, proteins and vitamins. In some cases, especially in artisanal food products, they dominate over the genus *Lactobacilli* and *Lactococci* (Pieniz et al., 2014; Porto et al., 2016). In fermented foods *Enterococci* can play an important role as starter cultures due to some metabolic characteristics related to the production of proteolytic and lipolytic enzymes, and the production of diacetyl from citrate, positively influencing the ripening of these cheeses (Sanlibaba and Senturk, 2018). In addition, they may act as biopreservatives due to the production of active bacteriocins against pathogens of relevance in cheeses, such as *Listeria* sp. and *Staphylococcus* sp., called enterocins (Franz et al., 2011). Particularly, the inhibition of *Listeria monocytogenes* has been an extremely important criterion in the selection and application of new starters due to the high mortality and recurrent presence of this pathogen in milk, milk derivatives products and their processing plants in Brazil and worldwide (Swaminathan and Gerner-Smidt, 2007; Barancelli et al., 2014).

Infections caused by food isolated *Enterococci* has not yet been proven, however, according to Giraffa et al. (2002), the high occurrence of VRE in foods make them reservoirs in the dissemination of antibiotic-resistant traits in the environment. They suggest a relation between the use of some antibiotics in livestock and humans, and them

becoming colonized by antibiotic resistant *Enterococci* via the food chain. For safety reasons, an important criterion is the absence of transferable antibiotic resistance genes in *Enterococci* that could be used as co-cultures or starter cultures in foods.

Considering the controversial properties of *Enterococci* sp. and current doubts about its safety status in the development of starters and/or adjunct cultures, the present study aims to evaluate the occurrence of this genus in Brazilian artisanal cheeses (n = 578) from five different regions of the country, as well as to evaluate their technological potential in relation to the production of enzymes and the production of bacteriocins against pathogenic microorganisms of relevance to the food industry. In addition, a brief evaluation of the safety aspects regarding the production of haemolysins and resistance to vancomycin was carried out.

2. Material and methods

2.1 Cheese sampling, LAB counting and isolation

Five hundred and seventy eight (n=578) Brazilian artisanal cheeses of eleven different types were analysed, being: Araxá, Cerrado, Serra da Canastra, Serro and Campo das Vertentes, from Southeast (n = 262), Coalho and Manteiga, from Northeast (n = 101), Serrano and Colonial, from South (n = 99), Caipira from Midwest (n = 108) and Marajó, from North (n=8). These cheeses weighed approximately 1 kg and were bought in different households or small grocery dairies, between July/2014 and February/2015. LAB count was performed according to Dowes & Ito (2001), using the pour plate method in two standard media: De Man, Rogosa and Sharpe agar (Merck, Darmstadt, Germany), specific for Lactobacillus sp. and agar M17 (Sigma-Aldrich, St. Louis, USA), specific for

Streptococcus and *Lactococcus*, overplayed with 1.2% bacteriological agar (Kasvi, Curitiba, Brazil) and incubated at 30 °C for 72 hours. The count was expressed as log CFU/g. Both mediums were supplemented with natamycin (50 mg/mL) (Danisco, Grindsted, Denmark) to avoid fungal contamination. Five characteristic colonies from MRS and M17 of each cheese were selected for purification and incubated at 30°C for 48 h again in the respective media. Gram (Gregersen, 1978) and catalase (3 % hydrogen peroxide) tests as well as morphologic observation, were conducted in order to eliminate non-LAB isolates (e.g. Gram-positive, catalase-negative and cocci or rod morphology). Cultures identified as LAB were kept in MRS broth (Acumedia, Neogen Corporation, Lansing/MI) with 30% glycerol at -80°C.

2.2 Molecular analysis

2.2.1 Enterococci selection

Based on the morphology (small and transparent colonies in MRS agar and in microscopic gram analysis), 372 strains isolated from M17 agar and 248 from MRS agar were submitted to PCR analysis in order to confirm their *Enterococci* sp. classification. The cultures were grown overnight in MRS broth at 37 ºC and streaked on MRS agar in order to obtain single colonies. After 48 h of incubation, one single colony were picked and streaked to the bottom of one 200 µL microtube and microwaved with maximum power for 2 min. Then, 25 µL of DNA free water was added, vortexed and centrifuged at 12,000 g for 10 min. Supernatant was frozen at -20 ºC for further use as template in a PCR analysis.

2.2.2 Enterococci sp. identification

For the amplification of enterococcal DNA, primers E1 (5' TCA ACC GGG GAG GGT 3') and E2 (5' ATT ACT AGC GAT TCC GG 3') binding to positions 632-646 and 1353-1369 (conserved regions of 16S rRNA genes) were used (Deasy et al., 2000). The DNA was amplified in a final volume of 25 µL using a PCR Master Mix solution (Promega, USA, Madison), both primers (at 20 µM each) and two microliters of each DNA sample isolated by the method described above. Amplification was performed for 30 cycles in a DNA Thermal Cycler (Westburg, Germany) by denaturing at 94 ºC for 1 min, annealing at 55 ºC for 1 min, followed by polymerization at 72 °C for 1 min. Eight microlitres of the PCR product was visualized by electrophoresis on a 1.5% (w/v) agarose gel using 1x TAE buffer containing about 750 ng/ml ethidium bromide. A 1000-bp ladder was run alongside the samples as a molecular weight marker. The gels were run for 2 h at 100 V and the DNA was visualized by UV transillumination. DNA bands of 1100 bp were considered positive for *Enterococci.* Reference strains were used as controls (*Enterococcus faecalis* ATCC 29212, *Enterococcus faecium* H17). Representative of positive PCR strains of each cheese were selected and sent to a Dutch hospital called Ziekenhuis Gelderse Vallei for further identification at species level by Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF MS).

2.3 Virulent factors

2.3.1 Haemolytic activity

Haemolytic activity was assessed by cultivating strains on blood agar (7% v/v sheep blood) for 48 hours at 37 ºC, according to the fabricant recommendations. Strains that produced green-hued zones around the colonies (α-haemolysis) or did not produce

any effect on the blood plates (γ-haemolysis) were considered nonhemolytic. Strains displaying blood lyses zones around the colonies were classified as haemolytic (β-haemolysis). Test was performed in duplicate.

2.3.2 Vancomycin resistance

All PCR positive strains isolated from 11 different types of Brazilian Artisanal Cheeses were grown overnight in MRS broth at 30 ºC and spotted onto the top of selective VRE plates (Brilliance VRE, Oxoid, UK; and CHROMagar VRE, Chromoagar, Paris) and incubated at 37ºC for 24 hours in order to access their ability to resist to vancomycin and carry transfer genes, according to the fabricant recommendations.

2.4 Technological properties

2.4.1 Proteolytic and lipolytic activity

Proteolytic and lipolytic capacity were assayed according to Sahraoui et al (2015), with modifications. For the determination of proteolytic activity, 2 µL of culture grown overnight (~ 10^8 CFU/mL) in MRS broth were spotted onto the surface of reconstituted skimmed milk agar (RSMA) medium containing 10% of milk (m/v) and 2% of agar (m/v). Proteolytic positive strains were identified upon the formation of clear zones around the cells. For the determination of lipolytic activity, 2 µl of the cultures grown overnight in MRS broth were poured onto plates containing lipolytic medium (meat peptone: 2.5 g / L; casein peptone: 2.5 g / L; yeast extract: 3 g / L; Agar-agar: 12g / L), added with 1% tributyrin and 0.0025% (w/v) red phenol as a coloured indicator for the production of butyric acid. The appearance of yellow halos around the cells indicated

positive activity. The results were expressed in high (+++), medium (++), low (+) activity and no (-) activity.

2.4.2 Bacteriocin activity against concerning pathogenic bacteria in dairy foods

The strains of *Enterococci* sp. were tested for their ability to produce bacteriocins against the following indicators: *Listeria monocytogenes* ATCC 35152, *Staphylococcus aureus* ATCC 19095, *Escherichia coli* ATCC 25922 and *Lactococcus lactis* NCD 0712, according to Charteris et al (1998), with modifications. Each indicator strain was cultured in BHI – Brain heart infusion (for pathogens) and M17 broth (for *Lc. Lactis*) and cell suspensions (10^9 CFU/mL) were prepared according to Sant'Ana et al. (2012). Ten microliters of frozen stock of each indicator were inoculated in 10 mL of Brain-Heart Infusion (for pathogens) or M17 (for *Lc. Lactis*) broth and incubated at 37 ºC for 24 h. The cell culture was centrifuged (4000 g; 10 min) and the pellets were washed twice with Phosphate Buffer Solution (pH 7.2). The OD of the cell suspension was adjusted to approximately 1 at 600_{nm} (≈ 10^9 CFU/mL). Fifty microliters of each indicator suspension were diluted in 50 mL of BHI (for pathogens) and M17 agar (for *Lc. Lactis*) and plated on petri dishes of 12 cm of diameter until solidification. Then, 3 uL of cell-free supernatant (obtained by filtering using a 96-microplate filter) of each LAB were spotted onto the top of each plate in order to assess their growth inhibiting activity against the pathogens, indicated by a clear zone around the spot. One plate containing just MRS medium added with purple bromocresol (80 mg/L) were also inoculated to ensure that the pH would not interfere in the inhibition.

2.5 5 Statistical Analysis

The data of technological potential and virulence factors were submitted to multivariate analysis of principal components using the software *Pirouette* vs. 3.11. Correspondence analysis was performed using *PAST vs. 3.26*.

3. Results and Discussion

3.1 Cheese sampling, LAB counting and isolation

Information on the prevalence, safety and quality aspects of *Enterococcus sp.* isolated from artisanal cheeses have been widely reported in the world, but limited information is available about Brazilian artisanal cheeses. Most of the available studies involving only Coalho, Canastra and Marajó cheeses (Andrade, 2009; Figueiredo, 2014; Porto et al., 2016). This study encompasses many samples from different types of Brazilian artisanal cheeses, collected throughout the country, as evidenced in Table 1.

[insert Table 1 here]

From 578 analysed samples of Brazilian artisanal cheeses, 300 (51.9 %) gave positive results for 16sRNA sequences indicating the presence of *Enterococcus sp.* (data not shown). When comparing between cheeses, the prevalence values ranged from 75 to 23.21%, for Marajó (from North region) and Araxá (Southeast region) cheeses, respectively.

The Marajó, Coalho and Manteiga cheeses presented the highest prevalence of *Enterococcus sp.* which may be related to the curd cooking step applied in their

processing, where the temperature ranges from 40 to 60 °C. *Enterococcus sp.* are able to resist at 60 ºC for 30 min (Giraffa and Rossetti, 2004; Carvalho, 2007). This step might thus select for this genus because of its thermal resistance when compared to another LAB (such as *Lactococcos* sp. and *Streptococcus* sp.). However, these values were lower than those reported by Templer & Baumgartner (2016) and Sanlibaba & Senturk (2018), which found a prevalence of 88.46 (23 of 26 samples analysed) and 99.1% (213 of 215 samples analysed) in samples of raw milk cheeses made in Italy and Turkey, respectively. In studies conducted with Brazilian cheeses from South and Southeast, the occurrence values were 100 and 83.3% (Furlaneto-Maia et al 2014, Gomes et al 2008) and 60.3% for Coalho cheese from Northeast region (Carvalho, 2007). Although the genus *Enterococcus sp.* is recognized as part of the natural microbiota of artisanal cheeses, high amounts of these microorganisms can be considered as indicative of contaminations due to precarious hygiene conditions during the production of artisanal cheeses. Contaminations could occur throughout the production process ranging from initial contamination of the milk to manufacturing e.g. manual pressing of cheeses and use of natural starters called as “pingo”, and storage.

Out of 11 cheeses a total of 1,512 presumptive identified lactic acid bacteria were isolated of which 576 (38.09 %) were confirmed as belonging to the genus *Enterococcus sp.* using 16sRNA amplification. The occurrence per cheese type is summarized in table 2 and Caipira, Marajó and Canastra cheeses had the highest occurrence values. To validate the 16sRNA amplification strains from each cheese were selected for further identification at species level by MALDI-TOF and the following species were found: *Enterococcus faecium* (58.73 %), *Enterococcus faecalis* (31.75 %),

Enterococcus durans (4.76 %), *Enterococcus hermanniensis* (1.59 %) and *Enterococcus gilvus* (1.59%).

[insert Table 2 here]

Enterococci are natural residents of human and animal intestinal tracts, but due to their high adaptability to various environmental conditions, they have a broad distribution in nature, being present in a wide range of food products, including cheeses (Lebreton et al, 2014). The most commonly encountered enterococcal species in the gut of mammals are *E. faecalis*, *E. faecium*, *E. hirae*, and *E. durans*, which comprises the same species we found in this study.

Our results are close to those reported by Fuka et al (2017) who found an occurrence of 53.8 % of *E. faecium*, 42.4 % of *E. faecalis* and 2.84% of *E. durans* in raw Istrian cheeses, but different from Templer et al (2016) who found *E. faecalis* as the predominant species (80.7%), followed by *E. faecium* (5.1%), and *E. durans* (11.7%) when analysing Appenzeller and Schabziger Raw Milk Cheeses. Studies performed by Andrade (2009), on the other hand, reported only a high occurrence of *E. faecium* (82.3 %) in Minas Artisanal Cheeses from Canastra. The predominance of *E. faecium* and *E. faecalis* over other *Enterococci* is in accordance with other studies on artisanal cheeses and dairy products, where high abundance of these two species has been reported (Franz et al, 1999, Mirhosseni et al 2018). The isolation of *E. hermanniensis* in this study (from Serrano cheese) is not common in milk and milk related products, being generally associated with meat products (Martin et al, 2009; Koort et al, 2009). The presence of *E. gilvus* (isolated from Minas Artisanal cheese from Canastra) is also not often reported in cheeses (Zago

et al., 2009). One study performed by Ohki et al (2018) reported the full genome sequence of one raw milk-isolated *E. gilvus* CR1 able to produce carotenoids. This indicates that artisanal cheeses might be interesting reservoirs to find new species of microorganisms with interesting functionalities from a biotechnological point of view.

3.2 Technological properties

In order to evaluate the quality and safety of *Enterococcus sp.* strains isolated from Brazilian artisanal cheeses made in five different regions as well as their potential for application in dairy fermentations, analyzes on extracellular enzyme (lipolytic and proteolytic) production and antagonistic activity against specific pathogenic microorganisms in cheese processing, such as *Listeria monocytogenes,* were performed. In addition, we assessed virulence factors such as haemolysin production and vancomycin resistance. From all 576 *Enterococcus sp.* strains isolated in this study, 26.81% were able to produce proteolytic enzymes in skim milk agar medium, 25.10% were able to produce lipolytic enzymes and 17.3% were able to inhibit the growth of *Listeria monocytogenes* only, not showing inhibitory effect against the other pathogens tested and *Lactococcus lactis* NCDO712 (Gasson 1980; Tarazanova 2017).

It is known that the production of extracellular enzymes by LAB plays an important role in ripened cheeses, contributing to their texture and flavor. The genus *Enterococcus*, however, exhibits low proteolytic and lipolytic activity, as reported in the literature (Bhardwaj et al, 2008) and this feature varies according to the origin and species of isolation. In one study performed by Joauani et al. (2015), only 9.1% of the *Enterococcus* strains studied were able to degrade casein, showing positive values in the proteolysis test and none of the studied strains showed lipolytic activity. A more recent

study reported a prevalence of 29.7 and 20.31% of proteolytic and lipolytic activity in *Enterococci* strains isolated from Cypriot Green Table Olives, respectively (Anagnostopoulos et al, 2018), which are closer to those of this study. However, because *Enterococcus sp.* constitutes a major part of the microbiota of artisanal cheeses, its role in the organoleptic characteristics of artisanal cheeses must be considered. Another factor that has been widely investigated is the search for starter cultures capable of producing antimicrobial substances, such as bacteriocins, in order to prevent growth of foodborne pathogen thus ensuring the quality and safety of dairy products. The production of enterocins by *Enterococcus* genus, is a wide-spread trait in raw milk and raw milk products (Mirhosseni et al, 2017). In Brazil, Alexandre (2002) found an occurrence of 25 % of *Enterococcus* sp. strains able to inhibit *Listeria monocytogenes*, demonstrating that artisanal cheeses from Brazil as good reservoir of new biopreservatives.

However, due to recent descriptions of *Enterococcus sp.* as opportunistic pathogen in hospital environments it is not classified as safe for human consumption, resulting in a controversial role in food. This leads to questions about safety, mostly due to the increasing amount of food-isolated VRE strains. In this study, 28.14% of the strains identified as *Enterococci* were able to grow in both chromogenic media specific for the detection of VRE strains and 56.84% shown haemolytic activity (11.98% and 44.87% of these classified as beta and alpha-haemolytic, respectively). These values were similar to those found by Sanlibaba et al (2018), who found values of 11.3% (24 of 213) of beta-haemolytic activity, but higher values of alpha-haemolytic activity (78.8%). Higher frequencies of alpha haemolysin than beta-haemolysin have also been observed in other studies (Tuncer, Ay and Tuncer, 2013; İspirli, Demirbaş and Dertli, 2017). In studies made

with Canastra cheeses, however, no strains of *Enterococcus sp.* were able to produce haemolysins (Andrade, 2009).

Regarded to vancomycin-resistance data, it was noticed that the fraction of *Enterococci* strains capable of growing in both medium containing the antibiotic vancomycin (at 60 mg/L) was 2.2 times higher for the brand Oxoid (63.88 %) than Chromoagar (28.14 %). Both values were considered high since the frequency of VRE in food should range from 0 to 25%, according to (J. S. Wang et al. 2014), which must be taken into consideration by the authorities of public health, as vancomycin is one of the few alternatives in treating enterococcal infections and the number of VRE has been increasing progressively in Brazil and worldwide (Furtado et al. 2005). The presence of VRE in food is of main concern due to their multidrug resistance to a large variety of antibiotics and hence their increasing relation to nosocomial infections. In addition, Gomes et al. (2008) suggest that the dissemination of antibiotic resistant strains may be related to food ingestion, since the exchange of genetic material (like plasmids, phages and conjugative transposons) between bacteria occurs predominantly in the gut by horizontal gene transfer (Franz et al 2011) but can also occur during cheese processing (Templer et al 2016). The presence of VRE strains in Brazilian artisanal cheeses is consequently alarming, making it a reservoir for human infections. A possible explanation for the antibiotic resistance of these strains is due the feeding of animals with low doses of antimicrobials, in certain conditions, in order to increase productivity by improving feed conversion and decreasing morbidity and mortality caused by infection. The glycopeptide avoparcin was first introduced for growth promotion in 1975 and confers cross-resistance to vancomycin, selecting for outgrowth of VRE strains, and as a consequence, VRE was

common in the intestinal flora of farm animals in Europe during the 1990s (Bager et al. 1997).

Figure 1 shows the percentage of *Enterococci* isolates showing high proteolytic; high and moderate lipolytic activity; ability to inhibit *Listeria monocytogenes* growth (applying sterile supernatant), haemolysin production; and ability to growth in both standardized VRE medium. These data were submitted to a correspondence analysis in order to verify if there is an influence of origin of isolation (cheese source) in the studied variables (Figure 2). It was possible to notice that Araxá, Colonial and Campos das Vertentes cheeses stands out due high percentages of β-haemolytic strains (40, 22.5 and 20.41 %, respectively), which is critical from a health point of view, once the usage of strains able to hydrolysate erythrocytes is forbidden in the composition of starts and/adjunct cultures in fermented foods (Sanlibaba et al., 2018). Cheeses from Campo das Vertentes microregion, Araxá and Serrano also present the highest percentages (61.22, 46.67 and 46.30 %, respectively) of *Enterococci* strains able to growth in VRE agar plates.

[insert Figure 1 here]

[insert Figure 2 here]

Since the use of strains presenting β-haemolytic activity and resistance to vancomycin is unacceptable in the application as starter/adjuncts cultures in food production, isolates from this study were separated into two groups, as shown in figure 3: non-beta-haemolytic and non-vancomycin resistant *Enterococcus sp.* (Figure 3.A) -

comprising 63.19% of the isolates - and beta-haemolytic and vancomycin resistant (able to growth in both VRE medium) (Figure3.B), per cheese type. It is interesting to notice the high percentage of bacteriocin producer strains in the group A, while the group B showed a higher percentage of isolates capable of producing extracellular enzymes.

[insert Figure 3 here]

Although the use of *Enterococci* in industrial production remains controversial, in many countries (especially in Greece, Italy, France, Spain and Portugal) they are considered essential for flavor development of fermented food, mostly in cheeses, once their natural presence in milk gives traditional cheese specific flavor notes (Foulquié Moreno et al., 2006). For that reason, the data were subjected to a multivariate analysis of principal components with binary data, as indicated in figure 4, in order to find strains with interesting features. It was possible to observe the formation of 9 groups of isolates (Figure 4.A) and relate them to the studied variables (Figure 4.B). The bacteriocin production explained the grouping of the strains belonging to group 1 and 2, and the high extracellular production of proteolytic enzymes in milk explained the formation of groups 6 to 9. Groups 3, 4 and 5 collected the isolates with high and/or moderate lipolytic activity as well as the production of virulence factors (resistance to vancomycin and beta-haemolytic activity).

[insert Figure 4 here]

The characteristics of the isolates belonging to groups 1 to 9 were gathered in Figure 5 for a better understanding of the phenotypic expression of each group. Figure 4 shows the fraction of isolates, per group, able of producing b-haemolysins and extracellular enzymes (lipolytic and proteolytic), inhibiting only the growth of *Listeria monocytogenes* ATCC 35152 and withstanding the presence of vancomycin in both tested mediums. Group 1 was notable for containing only isolates (12.92 %) capable of producing bacteriocins (enterocins) against *Listeria monocytogenes* ATCC 35152, showing potential to be applied as biopreservatives in milk and fermented products. In group 2, there are 3 strains (2QB_MRS_308, 1QB_M17_131, 2QB_M17_511), from Serro, Coalho and Serrano cheeses that are able to produce bacteriocin and lipolytic enzymes. In group 3, if we take all beta-haemolytic and vancomycin resistant strains out, we get 15 and 12 (27 strains) with high or moderate lipolitic activity. In group 6, all strains showed high proteolytic activitity: strains 1QB_MRS_355, 1QB_M17_334, 3QB_M17_227, 1QB_M17_426 and 1QB_M17_562, isolated from Campo das Vertentes, Colonial, Serrano, Caipira and Cerrado cheeses, repectively. The isolates belonging to groups 3, 4, 5, 7, 8 and 9 showed hemolytic activity and/or resistance to vancomycin and are not recommended to be applied in food processes. None of the tested strains were able to inhibit pathogenic bacteria other than *Listeria monocytogenes*, which may suggest that the enterotoxin belongs to the Class IIa: *Listeria*-active bacteriocins (Malik et al. 2012). Besides that, this specificity against *Listeria* sp. shows an interesting potential as probiotic and protective cultures for cheese manufacture, given their very limited antagonistic activity towards dairy starter cultures such as *Lactococcus* and *Streptococcus*, as indicated by Mirhosseini et al (2017). Bacteriocins can be used as biopreservatives by adding them directly to a food product, or by adding bacteriocinogenic LAB strains that

produce these peptides in situ. The latter application is particularly interesting for raw milk cheeses, where adding bacteriocin producing LAB strains could inhibit spoilage and foodborne pathogens. This would improving product safety (Cavicchioli et al., 2017) especially for cheeses that are consumed without further processing.

The genus *Enterococcus* sp., as well as other LAB, could occasionally be involved in clinical infections, but many strains may be considered safe for use in food or as probiotics. *Enterococcal s*trains have been isolated from artisanal cheese and their cultures have been proposed as starter in cheeses such as Cheddar and Fontina (C. de Andrade 2009). Established enterococcal probiotics include, for example, *E. faecium* SF68® (NCIMB 10415, produced by Cerbios-Pharma SA, Barbengo, Switzerland) and *E. faecalis* Symbioflor 1 (SymbioPharm, Herborn, Germany). Both strains are produced in form of pharmaceutical preparations specially used for the treatment of diarrhea and for which this application is regarded as an alternative to antibiotic treatment (Franz et al. 2011). The capability of *Enterococcus* to produce antimicrobial substances as bacteriocins, acetic acid and lactic acid, combined with high levels of gut colonization creates a competitive advantage against pathogens, however, as genetic exchange is particularly prevalent in the gut, the use of *Enterococci* as probiotics should be carefully considered from a safety point of view.

4. Conclusion

Our study highlighted the presence of *Enterococci* as a considerable part of the endogenous microbiota of Brazilian artisanal cheeses, mostly in Marajó, Coalho and Manteiga possibly due to the curd cooking step during the process. The species of

Enterococcus faecium, *Enterococcus faecalis*, *Enterococcus durans*, *Enterococcus hermanniensis* and *Enterococcus gilvus* were found. The high rate of resistence to vancomycin and haemolysins production should be taken into consideration due to the high frequencies of transmission of genes encoding for antibiotic resistance and virulence factors in GI tract. We were able to identify *Enterococci* (63.19%) with potential for application as starter cultures and/or biopreservatives in the production of fermented products derived from milk due to the absence of beta-haemolysin production and resistance to vancomycin. The application of protocols designed to analyse a large number of isolates and the mutivariate analysis of these data proved to be a good tool to access the phenotypic characteristics of the *Enterococci* strains tested and their variability. The strains from Serrano and Cerrado cheeses stands out due their bacteriocin (enterocin) inhibition against *Listeria monocytogenes* ATCC 35152, wich is a very useful criterion when sreening strains with probiotic potential. More studies are necessary in order to obtain reliable techonological data from an “in product” point of view.

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TABLES

Table 1. Prevalence of *Enterococcus sp.* in Brazilian artisanal cheeses

Cheese type	Region	Analyzed samples	Samples containing *Enterococci* sp.	Prevalence (%)
Marajó	North	8	6	75.0
Coalho	Northeast	78	56	71.8
Manteiga	Northeast	23	16	69.6
Serrano	South	48	32	66.7
Canastra	southeast	48	28	58.3
Campo das Vertentes	southeast	54	30	55.6
Colonial	South	55	28	50.9
Cerrado	southeast	54	25	46.3
Serro	southeast	50	21	42.0
Caipira	Midwest	108	45	41.7
Araxá	southeast	56	13	23.2
TOTAL		578	300	51.9

Table 2. Occurrence (in percentage) of PCR positive *Enterococci* sp. strains isolated from Brazilian Artisanal Cheeses, per cheese type and region.

Cheese type	Region	Number of isolates	*Enterococci* sp. (n)	%
Caipira	Midweast	241	111	46.06
Marajó	North	33	15	45.45
Canastra	Southeast	150	66	44.00
Coalho	Northeast	242	105	43.39
Campo das Vertentes	Southeast	130	56	43.08
Manteiga	Northeast	70	30	42.86
Colonial	South	110	41	37.27
Serro	Southeast	135	46	34.07
Serrano	South	122	36	29.51
Cerrado	Southeast	107	29	27.10
Araxá	Southeast	172	41	23.84
Total		1,512	576	

FIGURES

Figure 1. Percentage of *Enteroccci* sp., per cheese type, and their ability to produce enzymes (proteolytic and lipolytic), bacteriocins against *Listeria monocytogenes* ATCC 35152, hemolytic enzymes and vancomycin resistance. "Prot +++" = high proteolytic; "Lip+++" = high lipolytic, “Lip++” = Moderate lioplytic, “Bac+” = bacteriocin producer, “β-hem” = beta-haemolytic, “Vanc O+” = vancomycin resistance in OXOID agar, “Vanc CH+” = vancomycin resistance in CHROMO agar, "n" = number of isolates per cheese. The colors were applied in order to compare the fractions per raws.

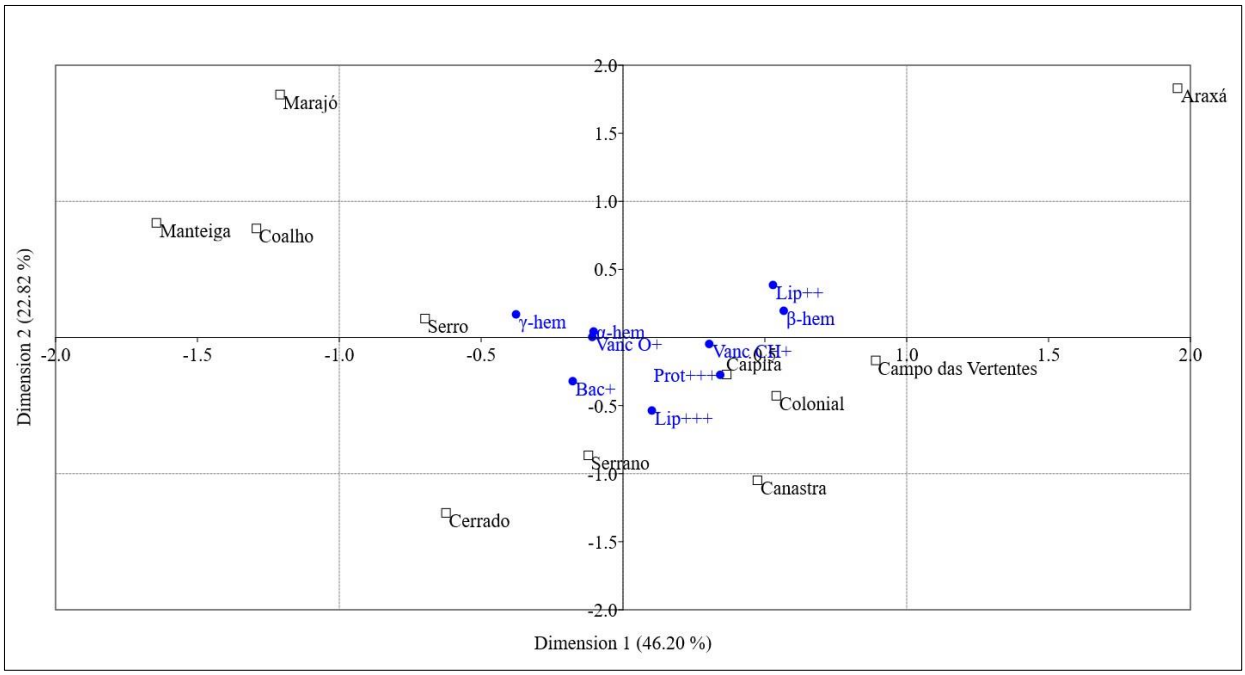

Figure 2. Map of correspondence analysis of *Enterococcus sp.* showing high proteolytic and/or moderate/high lipolytic activity and antilistrial activity. *Enterococcus sp.* strains were isolated from eleven cheese producing regions of Brazil (Marajó from North; Coalho and Manteiga from Northeast; Serrano and Colonial, from South; Caipira from Midwest and Minas Artisanal from Southeast, being classified according to the producing microregion as: Araxá, Cerrado, Campo das Vertentes, Canastra and Serro) and represented by square and empty lines. Their ability to produce enzymes (proteolytic and lipolytic), to inhibit *Listeria monocytogenes*, to produce haemolysins and to resist to vancomycin were represented by blue dots. "Prot+++" = high proteolytic; "Lip+++" = high lipolytic; “Lip++” = moderate lipolytic; “Bac+” = inhibition of *Listeria monocytogenes*; “β-hem” = beta-haemolytic; “γ-hem” =non-hemolytic; “α-hem” = alfa hemolytic; “Vanc O+” = growth in VRE medium from Oxoid; “Vanc CH+” = growth in VRE medium from Chromoagar.

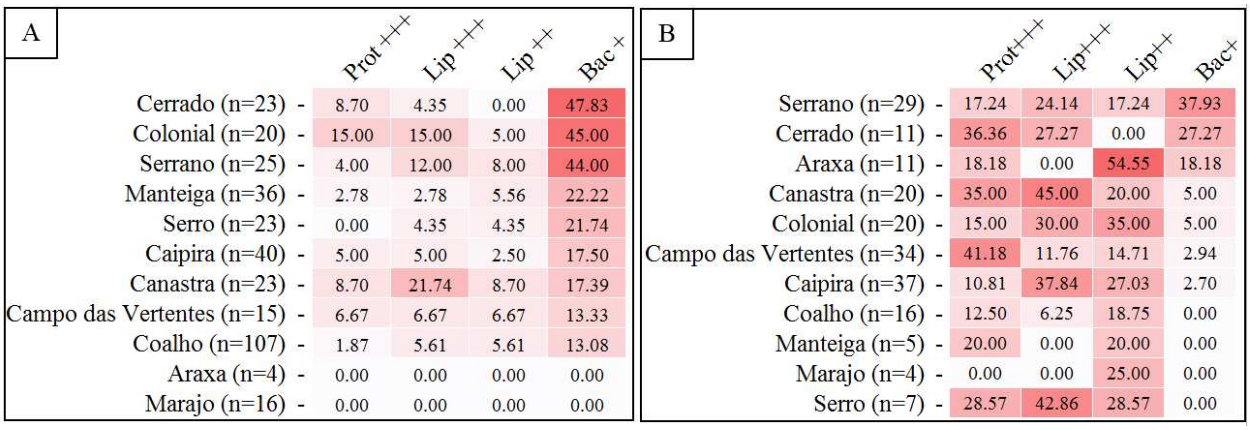

Figure 3. Percentage of non-beta-haemolytic and non-vancomycin resistant Enteroccci sp. (A) and of beta-haemolytic and vancomycin resistant Enteroccci sp. (B) per cheese type, and their ability to produce enzymes (proteolytic and lipolytic) and bacteriocins against *Listeria monocytogenes* ATCC 35152. "Prot +++"= high proteolytic; "Lip+++"=high lipolytic, Lip++=Moderate lioplytic, Bac+=bacteriocin producer., "n"=number of isolates per cheese.

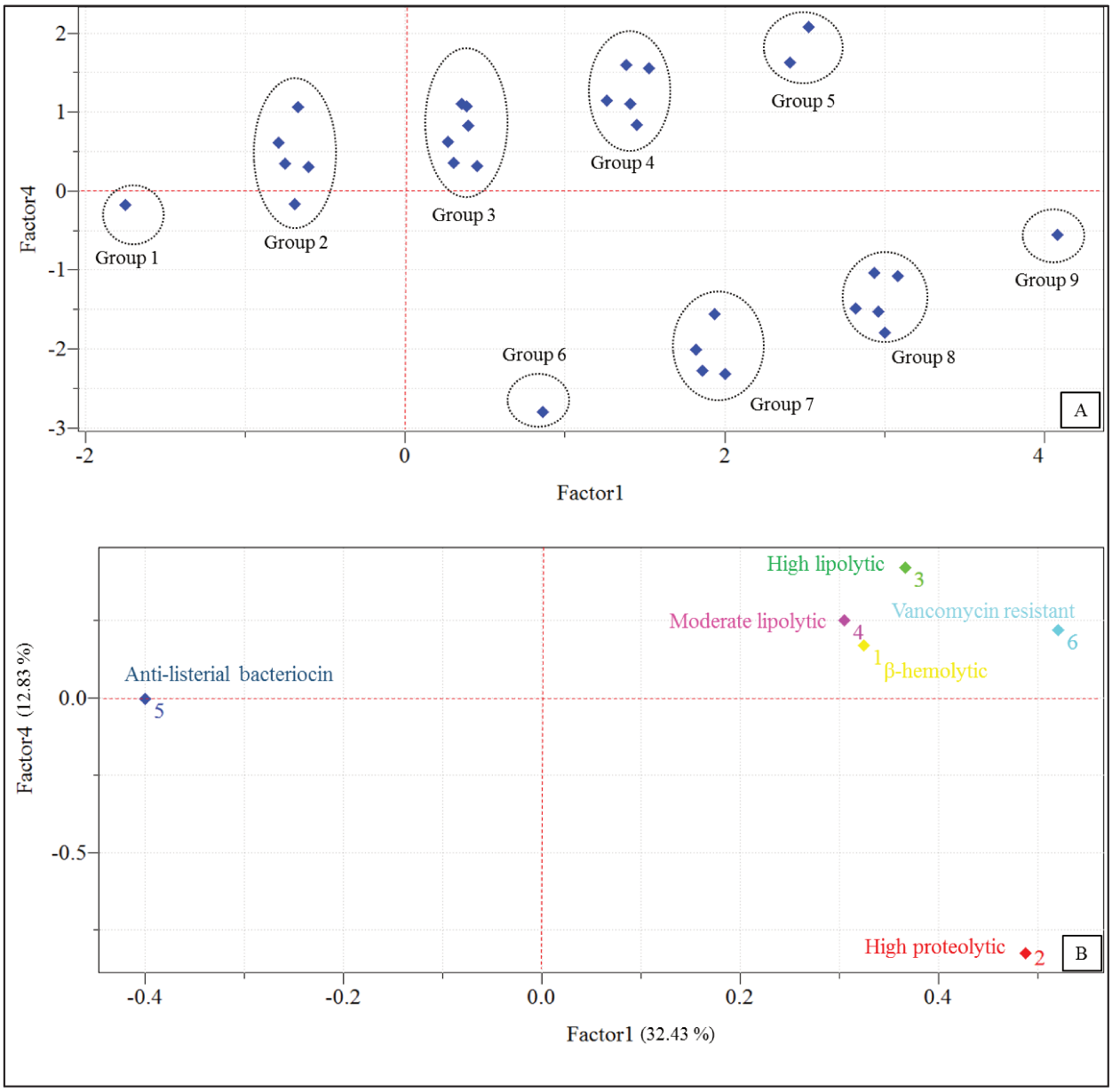

Figure 4. Principal component analysis (PCA) score plot of *Enterococcus*. sp strains isolated from Brazilian Artisanal Cheeses using the factors 1 and 4 (PC1 vs PC4).

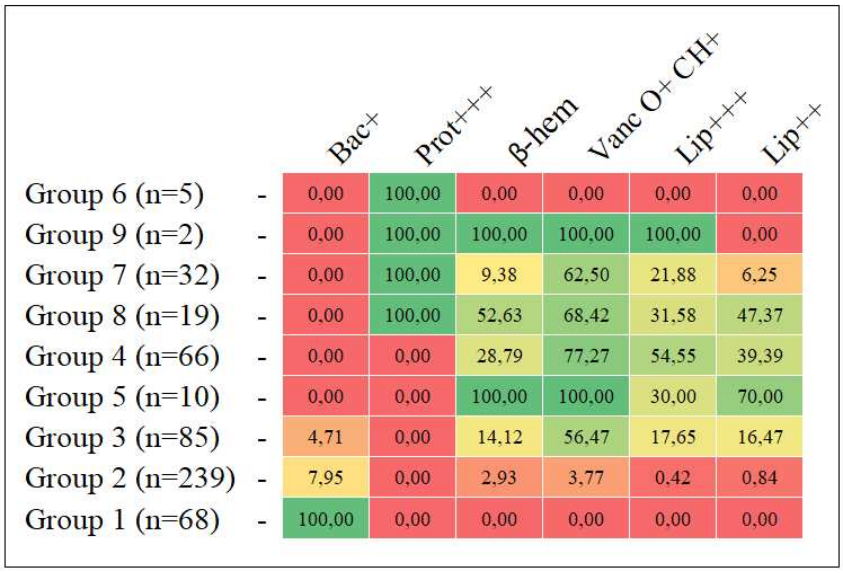

Figure 5. Phenotypic properties of PCA groups (Fig. 4) based on 576 *Enterocci* sp,. “Bac+” = bacteriocin producer; "Prot +++" = high proteolytic; “β-hem” = beta-haemolytic activity; "Lip+++" = high lipolytic, “Lip++” = Moderate lipolytic; “Vanc+”=resistence to vancomycin; "n" = number of isolates, per cheese group.

CAPÍTULO 3: Avaliação da formação de exopolissacarídeos, potencial aromático e biopreservativo de bactérias láticas não iniciadoras (NSLAB) isoladas a partir de queijos artesanais brasileiros – uma análise multivariada

Artigo formatado de acordo com as normas de submissão da revista “Food control”

Avaliação da formação de exopolissacarídeos, potencial aromático e biopreservativo de bactérias láticas não iniciadoras (NSLAB) isoladas a partir de queijos artesanais brasileiros – uma análise multivariada

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Abstract

Os queijos artesanais brasileiros (QAB) apresentam uma enorme diversidade microbiana, representada principalmente pelas bactérias acido láticas (BAL). Essas bactérias podem apresentar propriedades tecnológicas interessantes devido à sua capacidade de predominar e resistir aos processos de fermentação de leite e derivados. O objetivo deste estudo foi avaliar as propriedades biotecnológicas, biopreservativas e aspectos de segurança de relevância industrial de BAL provenientes de 10 tipos de queijos artesanais, comercializados nas 4 principais regiões do país (Nordeste, Sudeste, Sul e Centro-Oeste). Um total de 220 cepas pertencentes aos gêneros *Lactobacillus* sp., *Lactococcus* sp. e *Pediococcus* sp., previamente caracterizados com alta atividade enzimática (proteolítica e lipolítica) foram avaliadas em relação à sua capacidade de produzir diacetil (precursor de compostos aromáticos), exopolissacarídeos (a partir de diferentes fontes de açúcar), atividade antagônica e produção de substâncias bacteriocigênicas contra diferentes estirpes dos patógenos *Listeria monocytogenes* e *Staphylococcus aureus*. Observou-se que 131 isolados (59,6%) foram classificados como fortes (40,5%) e moderados (19,1%) produtores de diacetil; 28 (12,73%) isolados se destacaram por apresentarem forte formação de exopolissacarídeos a partir de diferentes fontes de açúcar: sacarose (3,2%), frutose (2,3%), lactose (2,3%) e glicose (6%); 94,1 e 95,9% dos isolados apresentaram atividade antagônica frentes os patógenos de *S. aureus* e *L. monocytogenes*, respectivamente, mas apenas 27 BAL (12,27 %) apresentaram resultados positivos no teste de produção de bacteriocinas, confirmando a origem proteica da substância antimicrobiana em questão. Nenhuma bactéria apresentou atividade hemolítica e 117 isolados foram classificados como seguros, devido a sua resistência intrínseca a até 4 antibióticos. Os resultados da análise de correspondência mostraram, para as linhagens testadas de BAL, que a primeira e a segunda dimensões englobaram com sucesso 45,27% e 22,17%, respectivamente, explicando 69,37% da variação total no conjunto de dados. Um grande aglomerado compreendendo queijos Araxa, Canastra e Campo das Vertentes e alta atividade de exopolissacarídeos da frutose, glicose e lactose e alta atividade de diacetil. As cepas dos queijos Coalho e Caipira se associam próximo à atividade bacteriocina. Este estudo demonstrou que os QAB são uma boa fonte de BAL selvagens com propriedades biotecnológicas de aroma e textura e biopreservativas interessantes para a aplicação como fermentos lácteos na formulação de produtos brasileiros com características similares às regionais, valorizando o mercado brasileiro nesta área.

Palavras-chave: Queijos artesanais brasileiros, potencial biotecnológico, bacteriocinas, culturas adjuntas, bactérias láticas não iniciadoras, segurança de alimentos, qualidade de alimento, análise multivariada de componetes principais.

1. Introdução

Os queijos artesanais brasileiros são de grande importância histórica, socio-econômica e cultural para as comunidades tradicionais brasileiras e se diferenciam devido à sua fabricação secular, passada de geração em geração, mantendo caracteristicas ímpares quando comparadas a produtos industrializados. A presença de uma microbiota endógena especifica para cada região de produção confere aos mesmos características organolépicas distintas de aroma, sabor e cor. Estes atributos são muito apreciados pela população local que consome este tipo de produto e tem chamado a atenção de pesquisadores e indústrias por ser um reservatório rico em micro-organismos com potencial biotecnológico e funcional a serem explorados. Esta biodiversidade engloba cepas de leveduras, fungos e principalmente bactérias láticas (BAL), provenientes do leite cru, do ambiente de produção (ex: utensílios, manipuladores, insetos) e, no caso dos queijos artesanais de Minas Gerais, da cultura endógena chamada “pingo”, definida como uma porção de soro fermentado originado do dessoramento de queijos produzidos no dia anterior, coletado em vasilhames para ser utilizado como fermento na produção do dia posterior (Souza and Dias, 2017; Campagnollo et al., 2018; Bachtarzi, Kharroub and Ruas-Madiedo, 2019; Kamimura et al., 2019).

As BAL se destacam por estarem amplamente distribuidas na natureza, sendo isoladas a partir de diversos produtos alimentícios de origem animal, vegetal e também do trato intestinal de seres humanos, o que as torna reconhecida pelo seu estatus GRAs (“Genrealy recognized as safe”). O domínio deste grupo de bactérias em relação aos demais deve-se ao seu metabolismo característico em processos fermentativos,

envolvendo a síntese de ácidos orgânicos (ácido lático, acético, propiônico) e redução do pH, produção de compostos antimicrobianos (como bacteriocinas, peróxido de hidrogenio, diacetil, CO_2, etc), promovendo a inibição de micro-organismos indesejáveis nos alimentos, atuando como bioconservantes. Além disso, algumas espécies de BAL, denomidas como "*Non Starter Lactic Acid Bacteria*" (NSLAB), desempenham papel fundamental no desenvolvimento de aroma e textura durante a maturação dos queijos, contribuindo para a qualidade e identidade destes produtos. Estas características envolvem os sistemas proteolítico e lipolítico destas bactérias, bem como a produção de exopolissacarídeos (ESPs) como mecanismo de defesa em condições de estresse, protegendo-as contra a dessecação e ataque de bacteriófagos (Giraffa, 2012; Bintsis, 2018; Pereira et al., 2019).

Os ESPs produzidos pelas BAL se destacam devido à sua capacidade de formar soluções altamente viscosas, mesmo em baixas concentrações, e à sua natureza pseudoplástica, contribuindo para a textura e reologia dos alimentos, atributos muito apreciados pelos consumidores. Nos queijos, eles atuam ligando-se às moléculas de água presentes na estrutura complexa da rede de caseínas, interagindo com proteínas e micélios presentes no leite, fortalecendo a estrutura do coágulo e diminuindo a sinerese durante a primeira etapa de acidificação e coagulação presentes na fabricação de queijos (Duboc and Mollet, 2001). De acordo com a composição de açúcares presentes na sua estrutura, os ESPs podem ser divididos em homopolissacarídeos -HoEPS (composto por apenas um tipo de monossacarídeo) e heteropolissacarídeos-HeEPS (composto de dois a oito tipos de monossacarídeos, repetidos). Assim, além dos atributos reológicos, os EPSs produzidos pelas LAB podem ser utilizados como fontes de oligossacarídeos e monômeros de açúcar, podendo ser aplicados como prebióticos, nutracêuticos,

adoçantes, humectantes, dentre outros (Zhennai Yang, 2000; Sanalibaba and Cakmak, 2016).

Estudos envolvendo o isolamento de BAL autóctones e sua posterior aplicação como culturas *starters* ou adjuntas na produção de queijos têm se intensificado ao longo dos anos objetivando reproduzir as características sensoriais peculiares dos queijos tradicionais, porém, de maneira mais segura, contribuindo para o reconhecimento destes quanto a sua origem geográfica. Além disso, a aplicação de BAL em alimentos como biopreservativos tem se tornado uma forte tendência, devido a maior eficácia destes em relação a conservantes artificais por meio da produção de bacteriocinas, extendendo a vida de prateleira e evitando o crescimento de patógenos em alimentos lácteos. As bacteriocinas, por definição, são proteínas ou complexos protéicos, geralmente de baixo peso molecular, sintetizadas pelos ribossomos das bactérias e geralmente ativas contra micro-organismos geneticamente próximos. Os processos de inibição envolvem a formação de poros, a degradação do DNA, inibição da síntese de peptidioglicanos , dentre outros (De Pasquale et al., 2019; Pereira et al., 2019; Skariyachan and Govindarajan, 2019; Todorov, 2019).

Assim, o objetivo do presente trabalho foi avaliar a produção de exopolissacrídeos a partir de diferentes fontes de açúcar (lactose, sacarose, frutose e glicose), a formação de diacetil como precursor de compostos aromáticos e a atividade bacteriocigênica de 220 cepas de *Lactobacillus* sp. autóctones isolados a partir de queijos artesanais brasileiros comercializados em qautro regiões do país a fim de verificar o seu potencial biotecnólogico e biopreservativo para aplicação em alimentos mais saudáveis e com caracteristicas regionais, a partir de uma análise multivariada. Também foram

avaliados a susceptibilidade a antibióticos e a presença de hemolisinas, a fim de verificar a segurança para a formulação de fermentos lácteos destinados ao consumo humano.

2. Material e métodos

2.1 Isolados de BAL

Um total de 220 batérias de *Lactobacillus* sp., isoladas a partir de queijos artesanais brasileiros, foram avaliadas neste estudo, compreendendo os seguintes queijos: Minas artesanal das regiões do Araxá (n=16), Campo das Vertentes (n=36), Canastra (n=22), Cerrado (n=2) e Serro (n=23), da região Sudeste; Coalho (n=53) e Manteiga (n=8), da região Nordeste; Caipira (n=39), da região Centro-Oeste; e Colonial (n=12) e Serrano (n=9), da região Sul. Todas elas apresentaram resultados positivos nos testes de produção de enzimas (lipolíticas e proteolíticas), resistência ao sal e a baixos valores de pH. Os isolados foram mantidos congelados (-80 ºC) em caldo MRS, adicionados de glicerol a 20 % (v/v).

2.2 Propriedades biotecnológicas

2.2.1 Produção de diacetil

A avaliação da produção de compostos aromáticos pelas BAL foi avaliada pela análise de diacetil, conforme King (1948). As bactérias foram inoculadas em caldo MRS e incubadas a 30 ºC. Após crescimento *overnight* (~ 10^8 UFC/mL), os isolados de BAL foram centrifugados a 4,000 rpm por 15 min. Os pellets foram ressuspendidos em água peptonada, inoculado em 10 mL de leite UHT integral na proporção de 1% e incubados a 30 ºC por 24 h. Então, 1 mL da cultura foi adicionada de 0,5 mL de solução α-naphthol a

1% (m/v) e, concomitantemente, 0,5 mL de solução de hidróxido de potássio a 16% (m/v), seguidos de incubação a 37 ºC por 10 min. A produção de diacetil foi indicada pela formação de um anel rosáceo nos tubos e classificada como fraca, média e forte de acordo com a intensidade da cor do anel formado.

2.2.2 Produção de exopolissacarídeos

A avaliação da produção de exopolissacarídeos (EXP) foi realizada conforme Jaouani et al (2015). As cepas de BAL foram crescidas em caldo MRS *overnight* e estriadas em meios modificados de ágar MRS, contendo 5% das seguintes fontes de caborno: glicose, sacarose, frutose e lactose (Dinamica, São Paulo, Brasil). Após serem incubadas a 30 ºC por 48-72h, as cepas capazes de formar colônias viscosas e limosas foram consideradas como ESP positivas, sendo classificadas de acordo com a altura do polissacarídeo: alta (> 5 mm), moderada (2 – 5 mm) e fraca (< 2 mm).

2.2.3 Atividade antagonista e verificação de substâncias bacteriocigênicas

Os testes de atividade antagonista foram realizados pela técnica de difusão em ágar, por meio da verificação de halos ao redor da colônia de BAL, indicando a inibição do crescimento dos patógenos, de acordo com metodologia de Harris *et al*. (1989) . Para este teste, microgotas de 5 µL da cultura de BAL crescida em caldo MRS *overnight* foram despejadas em placas de MRS ágar e incubadas por 18 horas a 30 ºC. Em seguida, 10 mL de meio Brain Heart Infusion (BHI) semi-sólido (contendo 0,85% de ágar) contendo o patógeno na concentração de 10^5 UFC/mL foram despejados sobre a placa e incubados a 37 ºC por 24 h. Os patógenos utilizados neste primeiro *screening*

foram as cepas padrões *Staphylococcus aureus* ATCC 19095 e *Listeria monocytogenes* ATCC 7644.

Para avaliação da produção de bacteriocinas, as cepas de BAL que apresentaram atividade antilisterial e antiestafilocócica foram crescidas em caldo MRS por 24 h a 30 ºC e submetidas a centrifugação a 4,000 g por 15 min a 4 ºC (Sorvall Legend XTR, Thermo Scientific, Alemanha). O sobrenadante livre de células (SBNT) foi coletado e o pH ajustado para 6-6,5 com NaOH 1 N. Em seguida, o SBNT foi submetido a um tratamento térmico a 70 ºC por 30 min e esterilizado por filtração com uma membrana de 0,22 µm. A atividade antilisterial e antiestafilocócica do sobrenadante foi avaliada pelo método "spot-on-the-lawn method", com modificações (Van Reenen, Dicks e Chikindas, 1998). Uma alíquota de 10 µl do SBNT foi despejada sobre a superfície de placas contendo 10 mL de agar bacteriológico 1,5%, recoberto com 5 mL de sobrecamada de BHI semi-sólido (adicionado de 0,85% de ágar) contendo o patógeno numa concentração de 10^6 UFC/mL. As placas foram incubadas a 37 ºC por 12 h e foi observada a formação de halos ao redor das microgotas do SBNT. A confirmação da produção de bacteriocinas foi feita pela avaliação da natureza proteica do composto antimicrobiano. Para este teste, o SBNT foi tratado (1h a 37 ºC) com as seguintes enzimas proteolíticas: α-chymotrypsin de pâncreas bovino tipo II, *Streptomyces griseus* protease tipo XIV, tripsina e proteinase K (todas provenientes da Sigma-Aldrich, St. Louis, MO, USA), solubilizadas em solução tampão fosfato a 20 mM (pH 7,0). Após o tratamento, o SBNT foi aquecido a 90 ºC por 5 min a fim de inativar as enzimas e testados pelo método "spot-on-the-lown", acima descrito, para avaliação de atividade antimicrobiana residual contra os patógenos supracitados. A ausência de halos após o tratamento enzimático indicou a presença de bacteriocinas. Para este teste de atividade bacteriocingênica foram utilizadas os

seguintes patógenos envolvidos em surtos alimentares: *S. aureus* produtores de enterotoxina (FRI S6 e FRI 361) e cepas de *L. monocytogenes* ATCC 3968 – serotipo 1/2b e ATCC 3973 – serótipo 4b, isoladas de queijo e leite cru, respectivamente. Todas as cepas foram gentilmente doadas pela Fundação Oswaldo Cruz (Rio de Janeiro/RJ/Brazil).

2.3 Avaliação da segurança

2.3.1 Antibiograma

A análise de antibiograma foi realizada em duplicata, com três repetições, de acordo com a técnica adaptada de susceptibilidade a antimicrobianos por difusão da droga em discos (Charteris et al. 1998). As amostras de BAL foram previamente cultivadas em caldo MRS, coletadas e lavadas com PBS. Os pellets foram então transferidos para tubos contendo 3,5 mL de solução salina (0,85% NaCl) até alcançarem 0,5 na escala McFarland (10^8 UFC/mL). Em seguida, utilizando-se esfregaços, os microrganismos foram inoculados sobre a superfície de placas de Petri contendo ágar MRS. Logo após, foram distribuídos os discos (Cecon, São Paulo, Brasil) contendo os antimicrobianos: ceftazidima (30µg), clindamicina (2 µg), ciprofloxacina (5 µg), eritromicina (5 µg), gentamicina (10 µg), oxacilina (1 µg), penicilina (10U), estreptomicina (30 µg), tetraciclina (30 µg) e vancomicina (30 µg). As placas foram novamente incubadas a 37ºC por 24 h. O controle de qualidade dos discos foi realizado com uma amostra de *Escherichia coli* ATCC 25922. Após a incubação foram feitas as leituras dos diâmetros dos halos de inibição. Os resultados foram submetidos a uma classificação qualitativa dos microrganismos como sensíveis, moderadamente sensíveis ou resistentes às drogas antimicrobianas testadas.

2.3.2 Hemólise

Para a avaliação da atividade hemolítica, as cepas (n = 220) de BAL foram crescidas em caldo MRS overnigth, inoculadas em ágar sangue (7 % v/v sangue de carneiro) e incubadas a 37 ºC por 48 h, de acordo com as recomendações do fabricante. As cepas que apresentaram halos em tons esverdeados ao redor da colônia (α-hemólise) ou não produziram nenhum efeito nas placas de sangue (γ-hemólise) foram classificadas em não-hemolíticas. As cepas que apresentaram halos transparentes (β-hemólise) ao redor da colônia, foram classificadas em hemolíticas.

2.4 Análises estatísticas

Os dados semi-qualitativos de potencial biotecnológico (produção de exopolissacarídeos, diacetil e bacteriocinas) e de avaliação da segurança (antibiograma e atividade hemolítica) foram submetidos a uma análise multivariada de correspondência (CA), utilizando o software PAST *vs. 3.26,* a fim de avaliar o impacto do tipo de queijo/região de isolamento no desempenho das cepas. Os isolados que apresentaram susceptibilidade a menos de 4 antibióticos foram selecionados para uma análise de componetes principais, utilizando o software Pirouette *vs. 3.11* para verificar as relações entre as características biotecnológicas e, assim, selecionar os grupos com os melhores resultados a serem aplicados em alimentos. Os dados foram convertidos em códigos (3, 2, 1, e 0, para alto, moderado, fraco e não produtores, respectivamente) e usados como *input data*.

3. Resultados

3.1 Identificação e origem das BAL

Das 220 bactérias láticas que apresentaram bons resultados nos testes de potencial tecnológico (reportado em estudo anterior) enviadas para identificação, apenas 179 foram identificadas a nível de espécie e 41 classificadas como pertencentes ao gênero *Lactobacillus* sp. A figura 1 mostra a quantidade total de cepas isoladas, distribuídas por tipo de queijo/região e a fração de cada espécie presente.

[inserir Figura 1 aqui]

Observa-se que a espécie de *Lactobacillus plantarum* foi a mais frequentemente isolada a partir dos queijos artesanais (47,73 %), seguida por *Lactobacillus brevis* (16,36 %), *Lactobacillus paracasei* (9,09 %), *Lactococcus lactis* (2,73 %), *Lactobacillus rhamnosus* (2,27 %), *Pediococcus pentosaceus* (0,91 %), *Lactobacillus curvatus* (0,45 %), *Lactobacillus paraplantarum* (0,45 %), *Lactococcus garviae* (0,45 %) e *Pediococcus acidilactici* (0,45 %). O queijo do tipo canastra destacou-se por apresentar 7 das 10 espécies detectadas e os queijos caipira e colonial foram os únicos que apresentaram as espécies de *Lactobacillus curvatus* e *Pediococcus acidilactici*, respectivamente. A maioria dos isolados foram provenientes da região Sudeste, a partir dos queijos do tipo Minas Artesanal (45,00%), sendo distribuídos por microrregião: Serro (n=23 isolados), Cerrado (n=2), Canastra (n=22), Campo das Vertentes (n=36) e Araxá (n=16). Em segundo lugar, as cepas de melhor potencial tecnológico foram provenientes da região Nordeste (27,73%), a partir dos queijos Coalho (n=45), Coalho light (n=9) e

Manteiga (n=7). A região Centro-Oste e Sudeste compreenderam 17,73 e 9,55 % dos isolados, provenientes dos queijos Caipira (n=39), Colonial (n=12) e Serrano (n=9), respectivamente.

3.2 Potencial tecnológico

As BAL capazes de produzir enzimas lipolíticas e proteolíticas, precursoras de compostos aromáticos presentes nos queijos, resistentes ao sal e, consequentemente, ao processo tecnológico de produção queijeira, foram escolhidas para dar continuidade às demais análises de potencial tecnológico. Estes 220 isolados de bactérias láticas foram submetidos às analises de formação de diacetil, produção de exopolissacarideos, atividade antagônica e produção de bacteriocinas. A figura 2 mostra o critério de classificação em alta (+++), moderada (++) e fracas (+); e a formação dos halos de inibição nos testes de produção de bacteriocinas sem tratamento com enzimas e a ausência dos halos após o tratamento com as enzimas proteolíticas, o que indica a natureza proteica da substancia inibidora. De acordo com a figura 2, a formação de halos foi indicada por um símbolo positivo (+) e a ausência de halos foi indicada por um sinal negativo (-). A indicação de produção de bacteriocinas em pelo menos um tipo de tratamento enzimático foi simbolizada como um “ok”.

[inserir Figura 2 aqui]

A Tabela 1 resume todos os dados destes testes para as diferentes espécies de isolados. Observa-se que 131 isolados (59,6%) foram classificados como fortes (40,5%) e moderados (19,1%) produtores de diacetil; 28 (12,73%) isolados se

destacaram por apresentarem forte formação de exopolissacarídeos a partir de diferentes fontes de açúcar: sacrose (3,2%), frutose (2,3%), lactose (2,3%) e glicose (6%); 94,1 e 95,9% dos isolados apresentaram atividade antagônica frentes os patógenos de *S. aureus* e *L. monocytogenes*, respectivamente, mas apenas 27 BAL (12,27 %) apresentaram resultados positivos no teste de produção de bacteriocinas, confirmando a origem proteica da substância antimicrobiana em questão.

[inserir Tabela 1 aqui]

As espécies de *Lb. brevis* e *Lb. rhamnosus* apresentaram moderada ou alta formação de diacetil e boa formação de ESPs a partir da sacarose; As espécies de *Lc. lactis* e *Lb. plantarum* se destacaram pela alta formação de ESPs a partir de sacarose e glicose e pelo maior número de cepas produtoras de bacteriocinas; As espécies de *Pediococcus acidilactici*, *Pd. pentosaceus* e *Lb. curvatus* apresentaram fraca formação de diacetil e fraca ou nenhuma formação de exopolissacarídeos a partir de sacarose, frutose e lactose; As espécies de *Lb. paraplantarum* e *Lc. garviae* também apresentaram baixa formação de diacetil, mas apresentaram moderada produção de ESP a partir de frutose e lactose; e moderada ou fraca produção de ESP a partir de glicose e sacarose As cepas de *Lactobacillus* sp. e *Lb. paracasei* apresentaram baixa formação de EPS a partir das diferentes fontes de açúcar e também apresentaram produção de bacteriocinas. Os resultados da análise de bacteriocinas estão apresentados na tabela 2, juntamente com as informações de espécie e origem dos isolados, por tipo de queijo, cidade, região e microrregião (no caso dos queijos Minas Artesanal). Os testes que apresentaram halos de inibição frente aos patógenos sem tratamento enzimático do

sobrenadante e que, após o tratamento enzimático indicou ausência de halos, foram considerados positivos, demonstrando que o agente inibidor apresenta origem proteica. Ao todo, houve a confirmação de produção de substâncias com potencial bacteriocigênico por 27 bactérias.

[inserir Tabela 2 aqui]

Dentro deste grupo apenas 1 isolado (1QB314), foi capaz de inibir o crescimento de todos os patógenos testados. Cinco isolados (2QB502, 1QB77, 3QB167, 1QB167, 1QB459) foram capazes de inibir duas cepas de *Listeria* testadas e *S. aureus* FRI S6. Um isolado (2QB422) foi capaz de inibir as duas cepas de *Listeria* testadas. O isolado 2QB383 foi capaz de inibir *L. monocytogenes* ATCC 3968 e *S. aureus* FRI S6. Três isolados (1QB352, 1QB371 e 1QB98) foram capazes de inibir apenas *L. monocytogenes* ATCC 3968. Onze (1QB147, 2QB77, 1QB113, 1QB128, 2QB147, 1QB127, 3QB361, 2QB81, 1QB117, 3QB350 e 3QB398) foram capazes de inibir apenas *L. monocytogenes* ATCC 3973. A bactéria 3QB216 inibiu apenas *S. aureus* FRI S6 e as bactérias 1QB52, 2QB446, 2QB350 e 3QB497 inibiram apenas *S. aureus* FRI 361. A maioria das cepas foram isoladas a partir de queijo Minas artesanal (n=11) de diferentes microrregiões, a saber: Serro (n=5), Campo das Vertentes (n=4), Araxá (n=1) e Canastra (n=1); seguidas dos queijos Coalho (n=6), Caipira (n=5), Colonial (n=3) e Manteiga (n=2) e foram identificadas como *Lb. brevis* (n=3), *Lb. paracasei* (n=1), *Lb. plantarum* (n=15), *Lc. lactis* (n=2), *Pd. acidilactici* (n=1) e *Lactobacillus* sp. (n=5). Os dados de potencial tecnológico (moderada e alta produção de diacetil e EPSs e produção de bacteriocinas)

foram submetidos a uma análise multifatorial de correspondência a fim de elucidar a sua relação com a origem de isolamento (tipo de queijo), conforme consta na figura 3.

[inserir Figura 3 aqui]

Os outputs da análise de correspondência mostraram que a primera e a segunda dimensão compreenderam 39,1 e 27,87 %, respectivamente, explicando 66,97 % da variância total presente no conjuto de dados, com uma diferença significativa entre as variáveis analisadas ($p < 0,05$). Houve um agrupamento dos isolados provenientes dos queijos Manteiga e Colonial em torno da variável B (produção de bacteriocina); Os isolados provenientes do queijo Serro e Cerrado se agruparam em torno da variável S e G (indicando alta ou moderada produção de ESPs a partir de Sacarose e Glicose); Os isolados provenientes dos queijos Serrano, Canastra e Campo das Vertentes se agruparam em torno das variáveis F e L (indicando produção alta ou moderada de ESPs a partir de Frutose e Lactose); E os isolados dos queijos Araxá, Coalho e Caipira se agruparam em torno da variável D (alta ou moderada produção de diacetil).

3.3 Avaliação da segurança

Em adição aos dados de potencial tecnológico, também foram realizados testes a fim de verificar a resistência dos isolados de BAL frente a antibióticos de uso clínico. A tabela 3 mostra os resultados dos testes de antibiograma frente aos antibióticos recomendados pela CLSI (2012). Nota-se que a maioria das BAL apresentaram resistência aos antibióticos gentamicina (94,55%), vancomicina (97,27%) e ciprofloxacina (81,36%) e que todos os isolados apresentaram resistência a estreptomicina. Os isolados

apresentaram sensibilidade a tetraciclina (95%), eritromicina (93,18%), ceftazidima (80,45%), penicilina (76,36%) e clindamicina (71,82%). Em relação a oxacilina, 40,91 % dos isolados apresentaram resistência e 45,45 % apresentaram sensibilidade.

[inserir Tabela 3 aqui]

A figura 4 mostra as frações de BAL, por espécies, e a sua resistência frente aos antibióticos testados, sendo divididas em três classes: 2 a 4; 5 a 7; e 8 a 10. A maioria dos isolados (53,18 %) apresentou resistência a menos de 5 antibióticos; 43,18 % apresentaram resistência a 5-7 e 3,64 % a mais de 8. Observa-se que apenas 3 isolados da espécie de *Lc. lactis*, 1 isolado de *Lb. plantarum* e 4 de *Lb. brevis* apresentaram resistência a mais de 8 antibióticos. Apenas os isolados resistentes a menos de 4 antibióticos foram escolhidos para a continuação das análises.

[inserir Figura 4 aqui]

A figura 5 mostra os resultados de análise de correspondência entre os resultados de antibiograma classificados como resistentes e a origem de isolamento dos micro-organismos. Nota-se que houve associação entre os isolados de queijo Coalho condimentado e Cerrado e a resistência a penicilina; Os isolados provenientes de queijo Araxá se agruparam em torno da variável TET (resistência a tetraciclina); Os isolados do queijo Serro se agruparam em torno das variável ERI, idicando maior número de cepas resistentes à eritromicina; Os isolados dos queijos Serrano e Colonial se agruparam em torno das variáveis CLI e CAZ, indicando maior número de isolados resistentes e

ceftazidima. Os isolados de queijo Caipira, Canastra, Campo das vertentes e Coalho light se agruparam em torno das variáveis OXA e CIP, indicando agrupamento de isolados capazes de resistir à oxacilina e ciprofloxacina. Nenhum isolado apresentou resultado positivo nos testes de hemólise em ágar sangue, sendo todos classificados como γ-hemolíticos, o que é importante para a segurança das mesmas na aplicação em formulações de alimentos.

[inserir Figura 5 aqui]

3.4 Análise de Componentes Principais (ACP)

Os dados de potencial tecnológico (formação de diacetil, produção de ESPs e de bacteriocinas) dos isolados de BAL resitentes a menos de 4 antibióticos (n=117) foram submetidos a uma análise de componentes principais a fim de separá-los, de acordo com suas principais características, conforme visualizado na figura 6. As componentes PC1 e PC2 contabilizaram 30,46 e 22, 11% da variância total, respectivamente. A partir desta PCA, foi possível observar a formação de três grupos distintos de bactérias (Fig 6.A, Fig 6.B e Fig 6.C), cujas caractéristicas estão elucidadas na Fig 6.D. O grupo C destacou-se pela alta (+++) produção de exopolissacarídeos a partir de frutose e lactose, o grupo A se destacou pela fraca produção de exopolissacarídeos (+) a partir de frutose e lactose e o grupo B representou os micro-organismos não produtores de exopolissacarídeos a partir destes dois açúcares, porém bons formadores de diacetil.

[inserir Figura 6 aqui]

Observa-se que houve uma associação entre a variável alta produção de diacetil (D+++) e a fraca formação de exopolissacarídeos a partir de glicose e sacarose, enquanto que a alta produção a partir destes açúcares (Sac+++ e Gli+++) se correlacionou com a fraca formação de diacetil (D+). A quantidade de isolados produtores de bacteriocinas foi maior no grupo A (n=9), seguida do grupo B (n=4), grupos que apresentaram fraca ou nenhuma produção de ESPs a partir da lactose e frutose, respectivamente. Observa-se também que houve uma relação entre o tipo de queijo e os grupos formados, sendo o grupo A relacionado com o queijo Minas Artesanal da região do Serro; o grupo B com os queijos Minas artesanal das regiões do Araxá, Campo das Vertentes e Canastra; e o grupo C com os queijos Serrano e Cerrado. Em relação a identidade dos isolados, apenas o grupo A apresentou a espécie *Lc. lactis*; apenas o grupo B apresentou a espécie *Lb. curvatus* e apenas o grupo C, a espécie *Lc. garviae*. As demais espécies (*Lb. brevis*, *Lb. paracasei*, *Lb. plantarum* e *Lb. rhamnosus*) estiveram presentes em todos os grupos, indicando que suas propriedades variam de acordo com a região de isolamento, o que indica uma variabilidade intra-espécie dependendo do tipo queijo de onde foram isoladas.

4. Discussão

O presente estudo visou avaliar a produção de exopolissacarídeos partir de diferentes fontes de açúcar, a formação de diacetil em leite, o potencial bacteriocigênico e fatores de virulência de 220 BAL do gênero *Lactobacillus* sp., *Lactococcus* sp. e *Pediococcus* sp. classificadas como NSLAB em estudo anterior, por apresentarem resistência ao sal e baixos valores de pH e à capacidade de produzir enzimas

proteolíticas e lipolíticas em meio extracelular. A diversidade de BAL em queijos artesanais deve-se à diversos fatores, como a raça, o tipo de nutrição do rebanho, o processo de fabricação envolvendo técnicas rudimentares e à microbiota autóctone presente no leite cru e no ambiente de processamento, que contribui significativamente para as diferenças sensoriais de aroma, cor e sabor, encontradas em produtos artesanais produzidos em diferentes regiões do país. O gênero *Lactobacillus* sp. apresenta maior incidência em estudos envolvendo a avaliação de características pro-tecnológicas envolvendo queijos artesanais brasileiros (Cabral et al., 2016; Kamimura et al., 2019a; Kamimura et al., 2019b).

Uma das principais características das BAL responsável pela formação de aroma em alimentos fermentados é a síntese de diacetil (2,3-butanedione), um dos produtos (CO_2, aldeído, ácido acético, etc) formados por bactérias heterofermentativas a partir de citrato por meio da via pentose monofosfato, presente na fermentação de hexoses. Neste estudo, 131 (59,6 %) isolados foram capazes de produzir diacetil a partir da lactose presente no leite, indicando sua potencial aplicação como culturas adjuntas na produção de queijos. Nos queijos, as bactérias inciadores (geralmente homoláticas) reduzem o pH do leite, devido ao acúmulo de ácido lático sintetizados a partir da lactose presente no leite; quando o pH atinge 4,6, ponto isoelétrico da caseína, esta precipita e forma o coágulo; Em paralelo, os baixos valores de pH facilitam o transporte de citrato presentes no leite para dentro das BAL precursoras de compostos aromáticos (diacetil, aldeidos), por meio da enzima citrato permease (Souza and Dias 2017). Vários estudos envolvendo BAL autóctones de queijos artesanais têm retratado a produção de diacetil (Agostini et al. 2018; de Almeida Júnior et al. 2015; Ferrari et al. 2016; Souza and Dias 2017; Taboada et al. 2014), porém, este estudo envolveu uma grande variedade de

queijos artesanais brasileiros, comercializados nas quatro regiões principais do país, indicando os queijos Coalho, Caipira e Minas da microrregião do Araxá, provenientes das regiões Nordeste, Centro-Oeste e Sudeste, como os maiores reservatórios de cepas produtoras de diacetil (Fig.3).

Outro fator importante na seleção de culturas *starter* ou adjuntas, é a capacidade de formar exopolissacarídeos a partir de diferentes fontes de açúcar, atuando como emulsificantes, espessantes, estabilizadores ou agentes de ligação à água, características importantes do ponto de vista industrial, devido à melhora da textura e reologia associada a este fenótipo (Sanalibaba and Cakmak 2016). No presente estudo, a maioria das BAL preferiram a glicose (33,64 %) e sacarose (28,18 %) como principais fontes de carbono para a alta produção de ESPs, avaliada de acordo com a capacidade de formação de limosidade da colônia e detectada visualmente (Fig.2). Este fenótipo é o principal método de triagem empregado no *screening* de cepas com alta capacidade de produzir EPS em escala laboratorial, por meio da inoculação em placas contendo meio sólido apropriado, suplementado com os açúcares a serem estudados. Este método vem sendo amplamente reportado na literatura e tem destacado a glicose como a fonte de carbono mais eficiente para a produção de EPSs e a grande variabilidade na síntese e composição dos EPSs, dependendo da fonte de carbono em questão (Malik et al. 2009; Bennama et al. 2012; Ishola and AdebayoTayo 2012; Paulo et al. 2012; Zeidan et al., 2017; Yuksekdag and Aslim, 2008; Oleksy and Klewicka, 2018). Neste estudo foi possível observar uma associação entre a alta e/ou moderada produção de ESPs a partir de sacarose e glicose e a baixa formação de diacetil pelas BALs (Fig. 6.D) do grupo B (Fig.6B), e vice-versa. Isso pode estar relacionado com a possível formação de homopolissacarídeos (constituídos de apenas uma unidade repetida de

monossacarídeo), que ocorre fora da célula por meio da secreção de enzimas extracelulares glicosiltransferases, que têm maior afinidade pela sacarose, formando frutanos e glucanos, consumindo assim o açúcar que seria destinado à via glicolítica intracelular (Sanalibaba 2016; Yang 2000).

A relação existente entre a alta produção de EPSs a partir da lactose e da frutose pelas BAL (Fig.6D) pelas BAL do grupo C, pode estar relacionada com a síntese de HeEPS (formados de dois a oito tipos de monossacarídeos), que ocorre no citoplasma da célula por meio da via W_{zx}/W_{zy}, mediada pela presença dos genes *eps*, e que utilizam a lactose e frutose como principais monossacarídeos doadores para a produção dos polímeros, sintetizadas pelo metabolismo central da célula e posteriormente secretados para o exterior da célula (Schmid, Sieber, and Rehm 2015; Zeidan et al. 2017). As espécies encontradas neste estudo estão de acordo com as mais conhecidas e estudadas até então (Oleksy and Klewicka 2018).

Neste estudo, a alta incidência de BAL (66,36 %) com alta formação de EPSs a partir de pelo menos um tipo de açúcar indica que os queijos artesanais brasileiros podem ser considerados reservatórios de cepas autóctones para a produção de fermentos lácteos a serem aplicados *in situ* como culturas adjuntas ou em conjunto com culturas *starters*, para a melhoria da reologia e textura de alimentos fermentados, além de contribuir com as propriedades funcionais destes. Vários estudos envolvendo EPS(+) BAL têm indicado a melhoria do aroma, textura e consistência em queijos, principalmente com baixo teor de gordura (light), devido à retenção das moléculas de água durante a formação do coágulo, diminuindo a atividade de água (Aw) de água e, consequentemente, a sinerese (Ayyash et al. 2018; Berthold-pluta and Garbowska 2019; Schmid, Sieber, and Rehm 2015; J. Wang et al. 2018; Zhang et al. 2015). A produção de

EPSs pelas BAL provenientes neste estudo podem ser, assim, uma alternativa para aumentar o redimento na produção dos queijos, chamando a atenção das indústrias para fontes mais baratas e naturais de biopolímeros, com apelo sustentável e com características regionais, indicando os queijos Serrano (região Sul) e Minas artesanal da Canastra e Campo das Vertentes (região sudeste) como os principais reservatórios de BAL EPSs (+) a partir de lactose e frutose, e os queijos Minas artesanal do Serro e do Cerrado a partir de glicose e sacarose (Fig.3). Além destas aplicações tecnológicas, a produção de EPSs vem sendo associada ao potencial probiótico *de Lactobacillus* sp., por permitir o aumento do tempo de residência ao longo do trânsito gastrointestinal dos hospedeiros, facilitando a colonização do cólon e desempenhando atividade anticarcinogênica, antiulcerosa, antiviral e redutora de colesterol, contribuindo positivamente para a saúde humana, podendo ser utilizados na formulação de alimentos funcionais fermentados (Lynch et al. 2018; N'tcha et al. 2016; Zannini et al. 2016).

Outro fator importante a ser considerado é a produção de antimicrobianos ativos pelas BAL contra cepas patogênicas, principalmente bacteriocigênicos. A falta de padronização encontrada na produção de alguns queijos artesanais e algumas deficiências encontradas durante o processamento, como casos de mastite no rebanho, falta de higiene na ordenha e durante a manufatura, condições inadequadas de armazenamento, escoamento e comercialização do produto final, diminuem a qualidade microbiológica dos mesmos. Vários estudos têm reportado a presença de *S. aureus* em queijos brasileiros, com contagens acima do permitido pela legislação ($> 10^3$ UFC/g), com valores de até 10^5 UFC/g (Brant, Fonseca, and Silva 2007; Kamimura et al. 2019; Tavares et al. 2019; Zaffari and Mello 2007). Estes dados são alarmantes, pois nestas concentrações pode haver a formação de enterotoxinas por *S. aureus*, representando

risco para a saúde dos consumidores. Segundo o Ministério da Saúde, foram registrados 2.350 surtos de doenças transmitidas por alimentos, e o 3º maior agente causador foi *S. aureus* (9,40 %). A grande incidência de *Listeria monocytogenes* (1,4–6%) em queijos artesanais semi-duros comercializados no país também é alarmante, principalmente para a população mais vunerável (mulheres grávidas, recém-nascidos, pacientes imunocomprometidos, idosos), na qual a taxa de mortalidade envolvendo casos de listeriose são altas (20-30 %). É importante destacar que a legislação recomenda a ausência deste patógeno em produtos lácteos (Raimundo et al 2013; Souza et al 2002; Silva et al, 1998; FAO/WHO, 2002; McLauchlin, Mitchell, Smerdon, & Jewell, 2004).

Diante disso, o presente estudo demonstrou altas taxas de inibição das BAL (96,36%) testadas contra os referidos patógenos nos testes antagônicos e a confirmação de antimicrobianos de natureza proteica (*bacteriocin-like substances*) após tratamento enzimático em 27 isolados, com espectro de inibição variável entre as espécies, com destaque para os isolados 1QB314, 2QB502, 1QB77, 3QB167, 1QB167, 1QB459, identificadas como *Lb. plantarum*, *Pd. acidilactici*, *Lc. lactis* e ao gênero *Lactobacillus* sp. que foram capazes de inibir pelo menos 3 das 4 cepas de patógenos testadas. A produção de bacteriocinas é bem difundida em bactérias láticas em alimentos lácteos e não-lácteos, principalmente os tradicionais e tem despertado a atenção de pesquisadores e das indústrias em busca de novos tipos de biopreservativos, com apelo para produtos mais naturais e com características regionais (Agostini et al. 2018; Cabral et al. 2016; Handa and Sharma 2016; Hermans et al. 2007; N'tcha et al. 2016; Samedi and Linton Charles 2019; dos Santos et al. 2015; Souza and Dias 2017). Neste estudo, os queijos Minas artesanal, Coalho e Caipira (Tab.2) se destacaram como boas fontes de BAL produtoras de substâncias bacteriocigênicas, podendo ser aplicadas na formulação de

alimentos, podendo contribuir tanto para a segurança quanto para a manutenção do *terroir* dos produtos artesanais, reduzindo os riscos existentes a partir do consumo destes queijos (Campagnollo et al. 2018). Além disso, a atividade bacteriocigência frente a patógenos de importância em alimentos também tem sido um critério na seleção de BAL com potencial probiótico. Mas para isso, elas devem ser avaliadas quanto à biosegurança, a fim de garantir a ausência de riscos após ingestão.

A avaliação da presença de hemolisinas (enzimas que provocam lise dos eritrócitos) e da resistência a antibióticos de uso clínico são alguns dos fatores avaliados a fim de verificar a virulência das BAL com potencial para aplicação como biopreservativos ou probióticos em alimentos, e a sua segurança no consumo. Neste estudo, houve uma grande taxa de resistência das BAL aos antibióticos gentamicina, vancomicina, ciprofloxacina e estreptomicina, o que está de acordo com outros estudos envolvendo bactérias láticas em produtos artesanais (Agostini et al. 2018; C. R. G. Andrade et al. 2014; Costa et al. 2013; Rodríguez-Alonso et al. 2009). A resistência a tais antibióticos, pertencentes aos grupos aminoglicosídeos (gentamicina e estreptomicina), quinolonas (ciprofloxacina) e glicopeptídeos (vancomicina) é considerada natural ou intrínseca ao gênero *Lactobacillus* sp., não apresentando risco de transferência de genes de resistência para outras bactérias (Charteris *et al*., 1998; Danielsen and Wind, 2003; Herreros *et al.* 2005). A resistência variável à oxacilina por 40,91% das BAL estudadas também não representa risco, pois também vem sendo amplamente reportada como intríseca em *Lactobacillus* sp., considerando que este antibiótico não está relacionado com a conjugação por plasmídeos (de Sant'Anna et al. 2017), apesar de fazer parte do grupo dos β-lactâmicos. Essa resistência pode estar associada com a impermeabilidade da parede celular das bactérias ou pela produção e ação de β-lactamases (Charteris et

al., 1998; Costa et al., 2013). Estes dados sugerem que nossas cepas podem ajudar a manter o equilíbrio natural da microflora intestinal de pacientes tratados com estes antibióticos.

Em contrapartida, as maiores taxas de sensibilidade alta ou moderada das BAL foram detectadas para os antibióticos tetraciclina, eritromicina e ceftazidima (> 80,45 %), seguidos de penicilina e clidamicina (71,82-76,36%), o que é desejável, tendo em vista que a resistência a estes antibióticos tem sido associada com a aquisição de genes por transferência horizontal (Testore *et al.*, 2002; Gevers *et al.*, 2003). O principal motivo que favorece a emergência de bactérias multiresistentes é o uso indiscriminado de antibióticos como promotores de crescimento na pecuária e no tratamento de doenças, sendo a cadeia de alimentos a principal rota de transmissão destas para os humanos (Reis et al. 2016). Assim, a susceptibilidade a antimicrobianos envolvidos na transferência de genes de resistência é crucial em cepas candidatas para uso como probióticos ou como *starter*, pois caso contrário, elas podem agir como reservatórios destes genes e transferí-los para outras bactérias no trato gastrointestinal dos hospedeiros (Handa and Sharma 2016).

Neste sentido, apenas as BAL (117/220) resistentes a menos de 4 antibióticos e sem perigo de transferência para outros micro-organismos foram consideradas para a ACP, considerando os dados semi-qualitativos das propriedades tecnológicas avaliadas, capazes de explicar 52,57% (PC1 + PC2) da variabilidade presente entre as bactérias. Esta grande porcentagem sugere que há uma distribuição homogênea entre as espécies estudadas, com diferenças significativas. Neste estudo, foi possível observar a formação de três grupos distintos entre si, separados de acordo com a habilidade de utilizar a lactose e a frutose para a alta (Fig.6C) ou baixa (Fig.6A) formação de exopolissacarídeos,

indicado pela presença de limosidade da colônia (Fig.2B), e o grupo das cepas capazes de formar diacetil a partir da lactose e de produzir substâncias bacteriocigênicas (Fig.6B). Houve uma grande variabilidade intra-espécie, indicando a influência da fonte de isolamento na performance dos isolados. Esta ferramenta apresentou assim, grande utilidade para a seleção racional de cepas autóctones presentes em queijos artesanais de diferentes regiões do país, e com grande potencial para serem aplicadas como culturas adjuntas capazes de melhorar a textura e a viscosidade de alimentos fermentados, de implementar o aroma e o sabor, com características inerentes ao tipo de queijo das quais foram isoladas, e de atuar como biopreservativos, dependendo da necessidade da indústria e, principalmente, dos pequenos produtores.

6. Conclusão

Os dados deste estudo corroboraram o potencial do mercado brasileiro para o uso de bactérias láticas endógenas provenientes de produtos regionais, em oposição à recorrente utilização de culturas iniciadoras comercalizadas por empresas estrangeiras, promovendo a formulação de produtos com características organolépticas similares aos de origem destas estirpes. Assim, as BAL isoladas neste estudo podem fortalecer o desenvolvimento de fermentos com propriedades balanceadas, para a fabricação de derivados lácteos funcionais diferenciados, diminuindo os custos de produção e aumentando a qualidade, a segurança e a valorização de alimentos lácteos brasileiros. Além disso, este estudo se destacou por ser o primeiro a envolver a bioprospecção tecnológica da microbiota de uma ampla variedade de queijos artesanais brasileiros, comercializados nas quatro principais regiões do país, evidenciando a grande

variabilidade intra-espécie nos dados apresentados, fortemente relacionada com a origem de isolamento, demonstrada por meio de métodos estatatísticos de análise multivariada.

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TABELAS

Table 1. Isolados de BAL e sua classificação em alta (+++), média (++) e fraca (+) produção de diacetil e de exopolissacarídeos a partir das seguintes fontes de açúcar: sacarose (Sac), frutose (Fru), lactose (Lac) e glicose (Gli); Inibição antagônica contra *Staphyloccocus aureus* (S a +) e *Listeria monocytogenes* (L m +) e atividade bacteriocingênica (Bac+) do agente inibidor, indicando sua natureza proteica. As cores representam o número de isolados (n) positivos para cada análise, por espécie.

LAB id	D+++	D++	D+	Sac+++	Sac++	Sac+	Fru+++	Fru++	Fru+	Lac+++	Lac++	Lac+	Gli+++	Gli++	Gli+	S a +	L m +	Bac
Lactobacillus paracasei (n=20)	11	2	4	0	8	9	1	0	14	1	0	14	0	5	11	20	20	1
Lactobacillus sp. (n=41)	17	8	11	0	8	27	0	7	26	0	7	26	3	8	24	37	38	6
Lactobacillus plantarum (n=105)	39	21	31	5	21	63	3	25	57	3	25	57	7	34	42	97	100	14
Lactococcus lactis (n=7)	3	0	1	1	2	2	0	4	3	0	4	3	0	3	4	7	7	2
Lactobacillus rhamnosus (n=5)	4	0	0	0	4	1	0	2	2	0	2	2	0	1	3	5	5	0
Lactobacillus brevis (n=36)	15	11	4	1	11	20	1	12	17	1	12	17	1	10	20	35	35	3
Lactococcus garviae (n=1)	0	0	1	0	1	0	0	1	0	0	1	0	0	1	0	1	1	0
Lactobacillus paraplantarum (n=1)	0	0	1	0	0	1	0	1	0	0	1	0	0	0	1	1	1	0
Pediococcus acidilactici (n=1)	0	0	1	0	0	1	0	0	1	0	0	1	0	0	1	1	1	1
Pediococcus pentosaceus (n=2)	0	0	2	0	0	2	0	0	1	0	0	2	0	1	1	2	2	0
Lactobacillus curvatus (n=1)	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0
Total (n=220)	89	42	57	7	55	127	5	52	121	5	52	122	11	63	107	207	211	27

Tabela 2. Resultados obtidos para a caracterização de isolados de bactérias lácticas (BAL) produtoras de bacteriocina (Bac +) contra *Listeria monocytogenes* ATCC 3968 (LM 3968), *Listeria monocytogenes* ATCC 3973 (LM 3973), *Staphylococcus aureus* FRI S6 (SA FRI S6), *Staphylococcus aureus* 361 (SA FRI 361). Os resultados positivos (definidos como "ok") para a produção de bacteriocina (Bac +) foram descritos da seguinte forma: halos inicialmente observados em NT (tratamento padrão - nenhuma enzima adicionada às placas de sobrenadante sem células [SSC]), indicando inibição de patógenos (+), que não foi mais observado (-) após o tratamento do SSC com enzimas proteolíticas α-quimotripsina tipo II de pâncreas bovino (α-Q), protease tipo XIV de *Streptomyces griseus* (Sg), tripsina (TRI) e proteinase K (PK), corroborando a natureza proteica do (s) composto (s) sintetizado (s) pelo isolado de BAL testado.

LAB id	Code	LM 3968						LM 3973						SA FRI S6						SA FRI 361						Cheese type	City	Region	Microregion
		NT	α-Q	SG	TRI	PK	Result	NT	α-Q	SG	TRI	PK	Result	NT	α-Q	SG	TRI	PK	Result	NT	α-Q	SG	TRI	PK	Result				
Lactobacillu brevis	2QB422	+	-	-	-	-	ok	+	-	-	-	-	ok	-	-	-	-	-	-	-	-	-	-	-	-	Caipira	Ribas do Rio Pardo - MS	Mato Grosso do Sul	-
Lactobacillus brevis	2QB446	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	ok	Caipira	Anhanduí - MS	Mato Grosso do Sul	-
Lactobacillus brevis	1QB117	+	+	+	+	+	-	+	-	-	-	-	ok	-	-	-	-	-	-	+	-	-	-	+	-	Coalho	Marabá - PA	Nordeste	-
Lactobacillus paracasei	1QB128	-	-	-	-	-	-	+	-	-	-	-	ok	-	-	-	-	-	-	-	-	-	-	-	-	Coalho light	Taipú - RN	Nordeste	-
Lactobacillus plantarum	2QB350	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	ok	Caipira	Jaraguari - MS	Mato Grosso do Sul	-
Lactobacillus plantarum	3QB350	-	-	-	-	-	-	+	-	-	-	-	ok	-	-	-	-	-	-	-	-	-	-	-	-	Caipira	Jaraguari - MS	Mato Grosso do Sul	-
Lactobacillus plantarum	1QB147	-	-	-	-	-	-	+	-	-	-	-	ok	-	-	-	-	-	-	-	-	-	-	-	-	Coalho	Cajazeiras - PB	Nordeste	-
Lactobacillus plantarum	1QB113	-	-	-	-	-	-	+	-	-	-	-	ok	-	-	-	-	-	-	-	-	-	-	-	-	Coalho	Cajazeiras - PB	Nordeste	-
Lactobacillus plantarum	2QB147	-	-	-	-	-	-	+	-	-	-	-	ok	-	-	-	-	-	-	-	-	-	-	-	-	Coalho	Cajazeiras - PB	Nordeste	-
Lactobacillus plantarum	1QB127	-	-	-	-	-	-	+	-	-	-	-	ok	-	-	-	-	-	-	-	-	-	-	-	-	Coalho	Bom Conselho - PE	Nordeste	-
Lactobacillus plantarum	1QB314	+	-	-	-	-	ok	+	-	-	-	-	ok	+	-	-	-	-	ok	+	-	-	-	-	ok	Colonial	Lacerdópolis - SC	Sul	-
Lactobacillus plantarum	3QB497	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	ok	Colonial	Carlos Barbosa - RS	Sul	-
Lactobacillus plantarum	3QB216	+	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	ok	+	-	-	-	+	-	Minas artesanal	Araxá - MG	Minas Gerais	Araxá
Lactobacillus plantarum	1QB352	+	-	-	-	-	ok	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Minas artesanal	São João del Rei - MG	Minas Gerais	Campo das Vertentes
Lactobacillus plantarum	3QB361	+	+	+	+	+	-	+	-	-	-	-	ok	+	+	-	-	+	-	+	-	+	-	+	-	Minas artesanal	São João del Rei - MG	Minas Gerais	Campo das Vertentes
Lactobacillus plantarum	1QB371	+	-	-	-	-	ok	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Minas artesanal	São João del Rei - MG	Minas Gerais	Campo das Vertentes
Lactobacillus plantarum	1QB77	+	-	-	-	-	ok	+	-	-	-	-	ok	+	-	-	-	-	ok	-	-	-	-	-	-	Minas artesanal	Serro - MG	Minas Gerais	Serro
Lactobacillus plantarum	2QB77	-	-	-	-	-	-	+	-	-	-	-	ok	-	-	-	-	-	-	-	-	-	-	-	-	Minas artesanal	Serro - MG	Minas Gerais	Serro
Lactobacillus plantarum	3QB98	+	-	-	-	-	ok	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Minas artesanal	Serro - MG	Minas Gerais	Serro
Lactobacillus sp.	1QB459	+	-	-	-	-	ok	+	-	-	-	-	ok	+	-	-	-	-	ok	-	-	-	-	-	-	Caipira	Jaraguari - MS	Mato Grosso do Sul	-
Lactobacillus sp.	3QB167	+	-	-	-	-	ok	+	-	-	-	-	ok	+	-	-	-	-	ok	-	-	-	-	-	-	Manteiga	Cajazeiras - PB	Nordeste	-
Lactobacillus sp.	2QB383	+	-	-	-	-	ok	+	+	-	-	+	-	+	-	-	-	-	ok	-	-	-	-	-	-	Minas artesanal	São João del Rei - MG	Minas Gerais	Campo das Vertentes
Lactobacillus sp.	3QB398	-	-	-	-	-	-	+	-	-	-	-	ok	-	-	-	-	-	-	-	-	-	-	-	-	Minas artesanal	Medeiros - MG	Minas Gerais	Canastra
Lactobacillus sp.	2QB81	-	-	-	-	-	-	+	-	-	-	-	ok	-	-	-	-	-	-	-	-	-	-	-	-	Minas artesanal	Serro - MG	Minas Gerais	Serro
Lactococcus lactis	1QB167	+	-	-	-	-	ok	+	-	-	-	-	ok	+	-	-	-	-	ok	-	-	-	-	-	-	Manteiga	Cajazeiras - PB	Nordeste	-
Lactococcus lactis	1QB52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	ok	Minas artesanal	Serro - MG	Minas Gerais	Serro
Pediococcus acidilactici	2QB502	+	-	-	-	-	ok	+	-	-	-	-	ok	+	-	-	-	-	ok	-	-	-	-	-	-	Colonial	Carlos Barbosa - RS	Sul	-

Tabela 3. Número de isolados de bactérias láticas resistentes aos antibióticos testados na análise de antibiograma, onde EST: estreptomicina (30 µg), VAN: vancomicina (30 µg), GEN: gentamicina (10 µg), CIP: ciprofloxacina (5 µg), OXA: oxacilina (1 µg), CLI: clindamicina (2 µg), PEN: penicilina (10 U), ERI: eritromicina (5 µg), TET: tetraciclina (30 µg) e CAZ: ceftazidima (30µg).

LAB specie	1.GEN			2.PEN			3.CLI			4.TET			5.VAN			6.CIP			7.ERI			8.EST			9.OXA			10.CAZ		
	R	MS	S	R	MS	S	R	MS	S	R	MS	S	R	MS	S	R	MS	S	R	MS	S	R	MS	S	R	MS	S	R	MS	S
Lactobacillu brevis (n=36)	31	3	2	15	6	11	17	5	11	1	1	34	34	-	2	33	2	1	1	-	35	36	-	-	24	2	7	13	6	14
Lactobacillus curvatus (n=1)	1	-	-	-	-	1	-	-	1	-	-	1	1	-	-	1	-	-	-	-	1	1	-	-	-	-	1	-	-	1
Lactobacillus paracasei (n=20)	20	-	-	1	3	15	-	2	14	-	-	19	18	-	2	8	3	3	-	-	20	20	-	-	7	1	9	5	-	14
Lactobacillus paraplantarum (n=1)	-	-	1	1	-	-	-	-	1	-	-	1	1	-	-	-	-	1	-	-	1	1	-	-	1	-	-	1	-	-
Lactobacillus plantarum (n=105)	100	-	5	15	25	55	19	13	67	-	-	105	103	-	2	96	1	6	4	1	99	105	-	-	38	5	47	7	3	95
Lactobacillus rhamnosus (n=5)	5	-	-	-	-	5	-	-	5	-	-	5	5	-	-	3	-	-	1	-	4	5	-	-	-	2	3	-	3	2
Lactobacillus sp. (n=41)	40	-	1	1	9	30	3	5	30	6	-	35	41	-	-	27	3	6	4	-	36	41	-	-	15	-	19	6	1	31
Lactococcus garviae (n=1)	1	-	-	-	-	1	-	-	-	-	-	1	1	-	-	1	-	-	-	-	1	1	-	-	-	-	1	-	-	1
Lactococcus lactis (n=7)	7	-	-	3	2	2	4	-	3	3	-	4	7	-	-	7	-	-	3	-	4	7	-	-	3	-	2	3	-	4
Pediococcos pentosaceus (n=2)	2	-	-	-	1	1	1	-	1	-	-	2	2	-	-	2	-	-	-	-	2	2	-	-	1	-	1	-	-	2
Pediococcus acidilactici (n=1)	1	-	-	-	-	1	1	-	-	-	-	1	1	-	-	1	-	-	-	1	-	1	-	-	1	-	-	1	-	-
TOTAL (N=220)	208			36			45			10			214			179			13			220			90			36		

FIGURAS

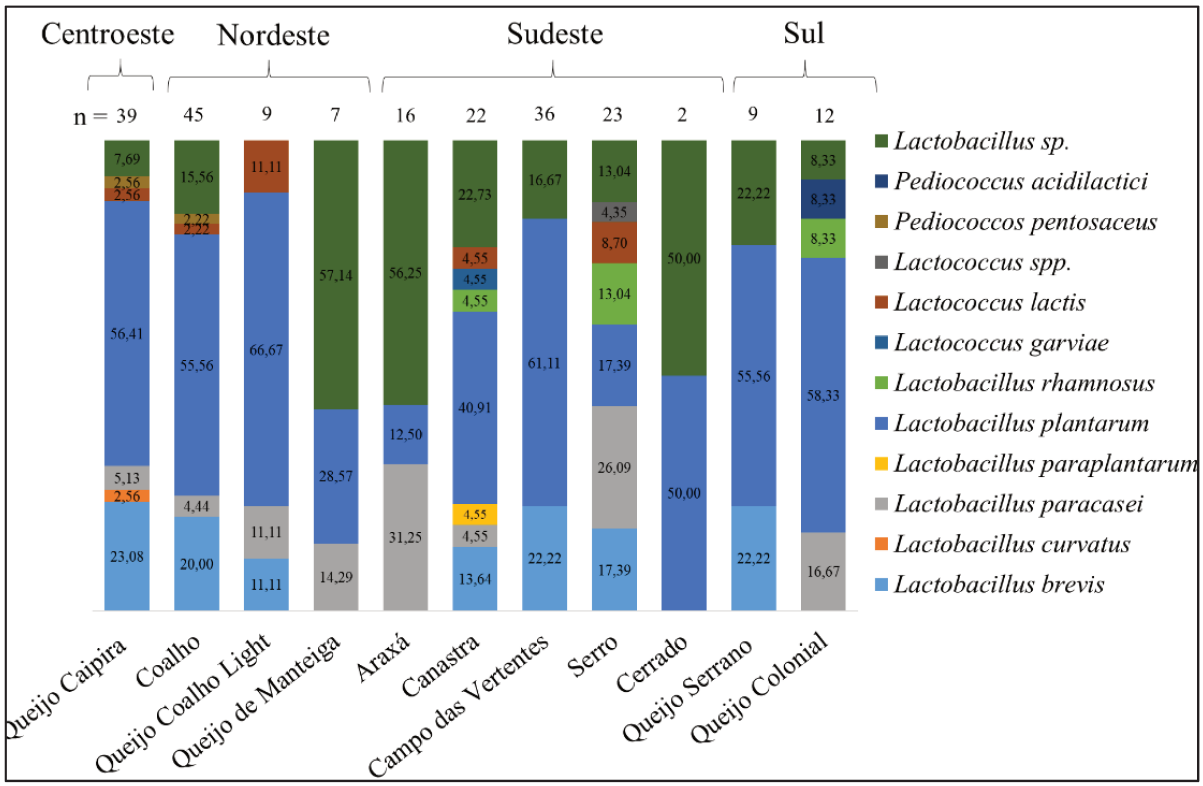

Figura 1. Diversidade de bactérias láticas (em porcentagem) encontradas em queijos artesanais brasileiros de diferentes regiões do Brasil. “n” = número de isolados de BAL com potencial tecnológico presentes em cada tipo de queijo.

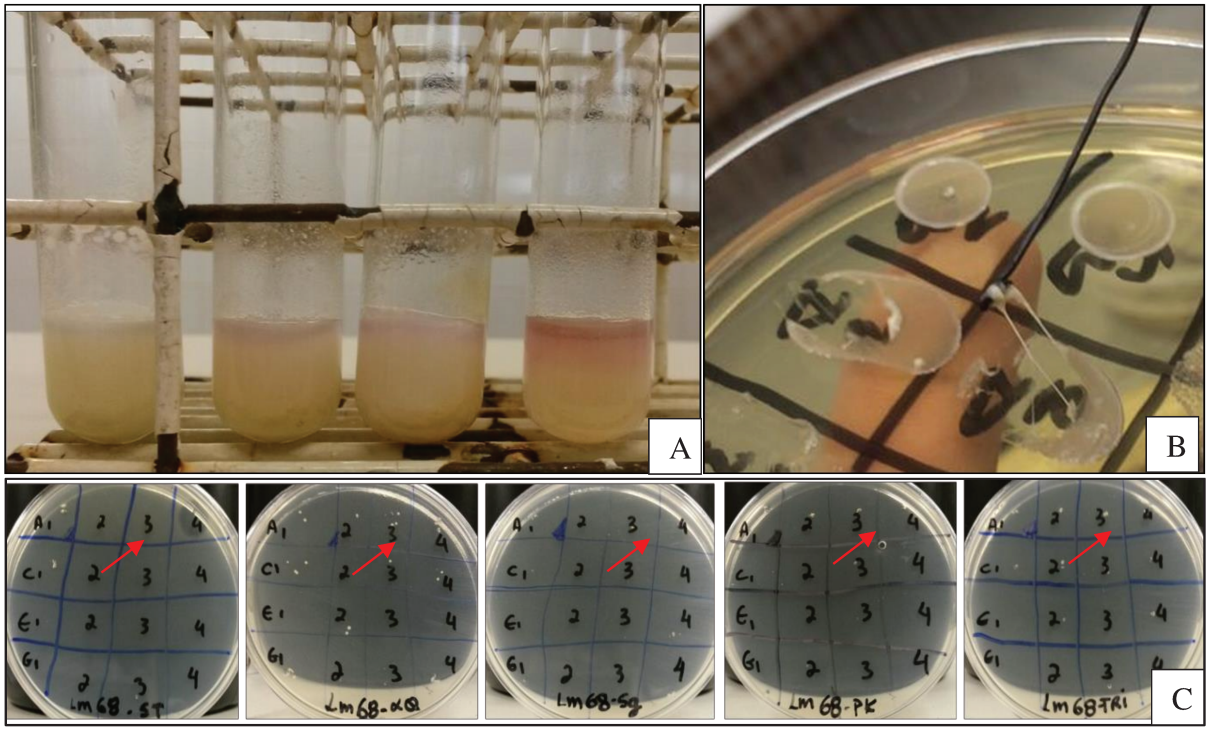

Figura 2. A) Análise de produção de diacetil, divididas em forte (+++), média (++), fraca (+) e não produtora (-) de diacetil, da esquerda para a direita, conforme intensidade rosácea; B) Análise de produção de exopolissacarídeos, com colônia produtora de limosidade classificada como alta (+++), indicando tamanho do polímero > 5 mm; C) Resultado da análise de bacteriocinas considerado positivo: O halo presente na placa ST (sem tratamento com enzima), indicando inibição do patógeno *Listeria monocytogenes* 68 (Lm 68) desaparece após o tratamento enzimático com as enzimas α-chymotrypsin (α-Q) de pâncreas bovino tipo II, *Streptomyces griseus* protease tipo XIV (Sg), tripsina (Tri) e proteinase K (pK), indicando a origem proteica da mesma. Cada quadrante da placa indica um microorganismo diferente.

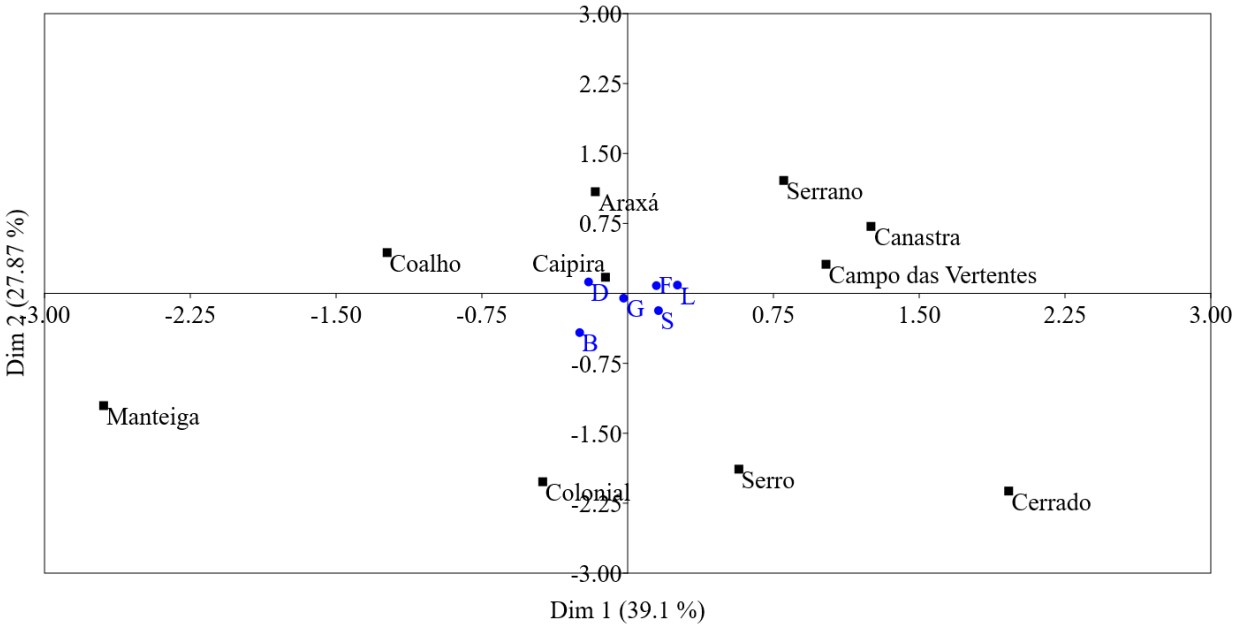

Figure 3. Map of correspondence analysis of lactic acid bacteria (LAB) isolated from Brazilian artisanal cheeses (BAC). LAB strains were originated from five producing regions of Brazil (Caipira, Coalho, Colonial, Manteiga, Araxá, Campo das Vertentes, Canastra, Cerrado, Serro and Serrano) and represented by solid squares. Bacteriocin (B), Diacetil (D) and exopolyssacaride production from Frutose (F), Lactose (L), Sacarose (S) and Glicose (G) are represented by solid blue circles and classified into moderate (++) and high (+++) activities.

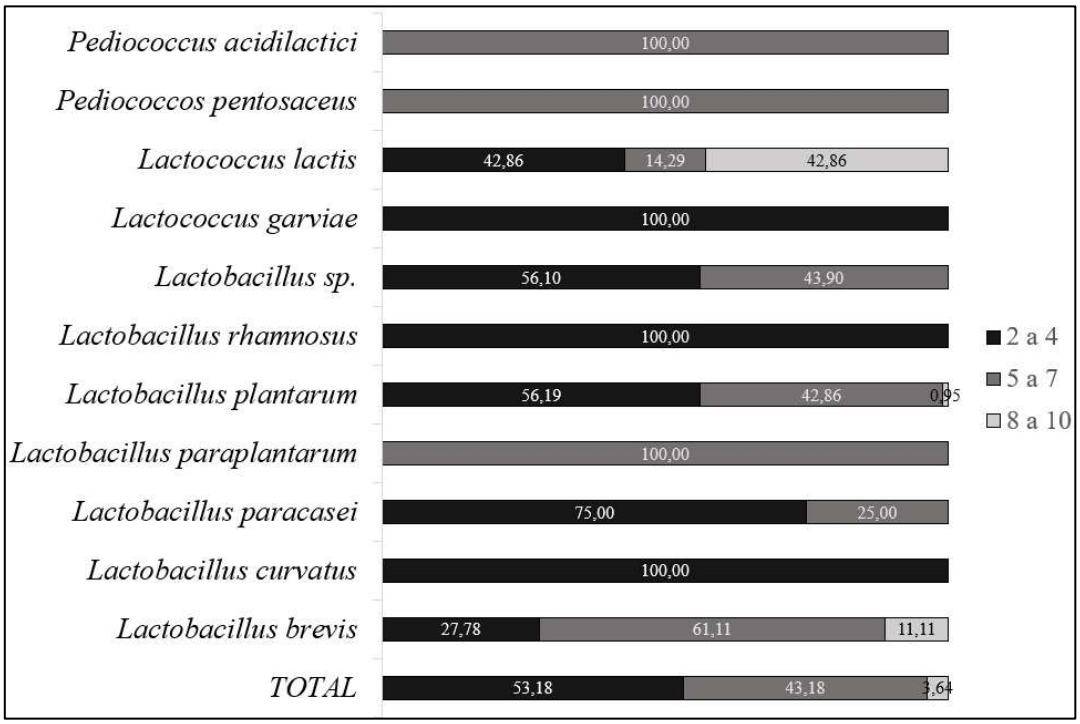

Figura 4. Classificação das BAL, por espécie, quanto à sua resistência a antibióticos: de 2 a 4, de 5 a 7 e de 8 a 10 antibioticos.

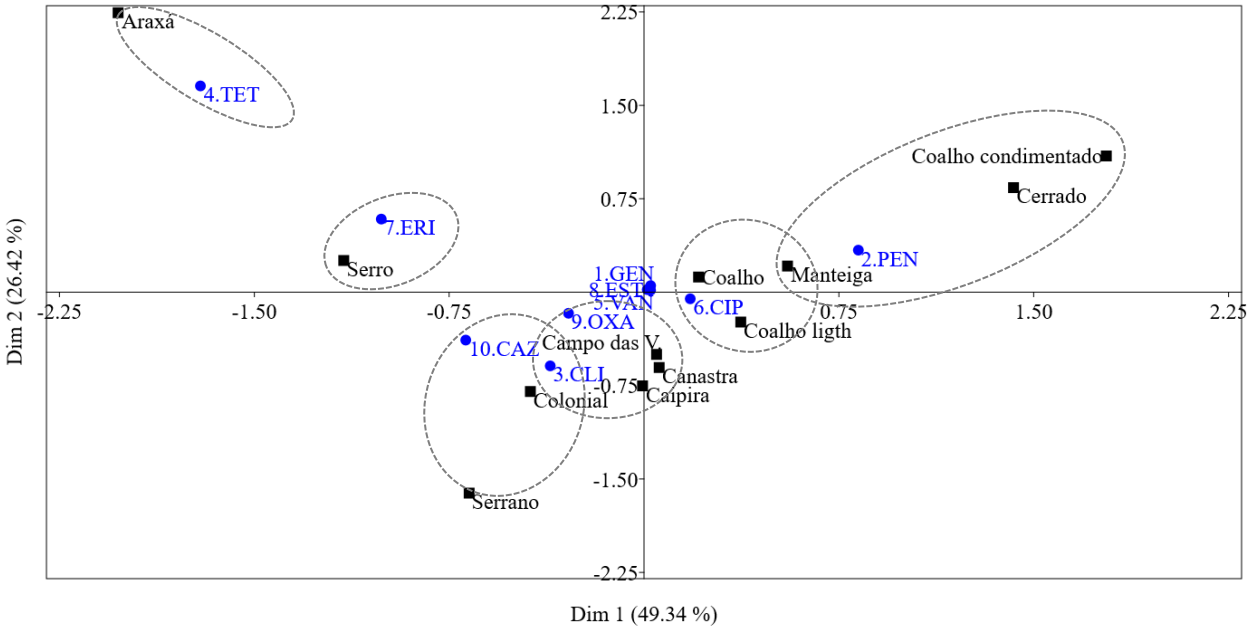

Figura 5. Mapa de análise de correspondência de bactérias acido láticos (BAL) isoladas a partir de diferentes tipos de queijo artesanais (representados por círculos sólidos pretos) e capazes de resistir aos testes de antibiograma pelo métodos de difusão em disco aos seguintes antibióticos: EST: estreptomicina (30 µg), VAN: vancomicina (30 µg), GEN: gentamicina (10 µg), CIP: ciprofloxacina (5 µg), OXA: oxacilina (1 µg), CLI: clindamicina (2 µg), PEN: penicilina (10 U), ERI: eritromicina (5 µg), TET: tetraciclina (30 µg) e CAZ: ceftazidima (30µg).

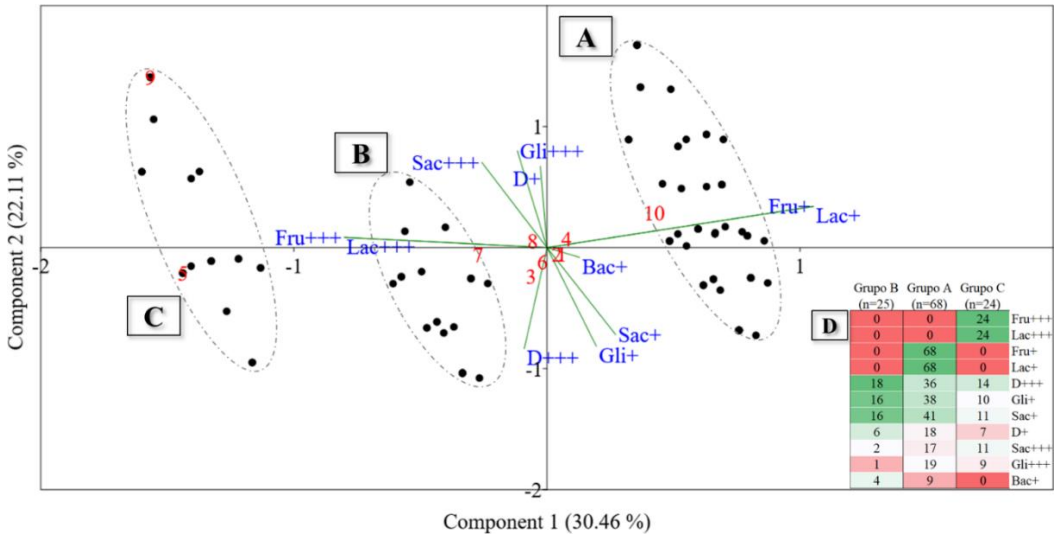

Figura 6. A) Principal component analysis (PCA) score plot of technological properties obtained from 117 LAB isolates and formed groups (Groups A, B and C); B) Resulting groups are shown according to microorganisms' phenotypic features regarding diacetyl formation (D) and exopolysaccharide production from fructose (Fru), lactose (Lac), sucrose (Suc), and glucose (Glu) as main carbon sources (both expressed as weak [+] or high [+++]). Finally, (n) represents the number of LAB isolates that presented the above-mentioned characteristics.

CAPÍTULO 4: Probiotic and safety assessment of wild NSLAB *Lactobacilli* sp. isolated from Brazilian Artisanal Cheeses produced in the main regions – from screening to *in product* approach

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Probiotic and safety assessment of wild NSLAB *Lactobacilli* sp. isolated from Brazilian Artisanal Cheeses produced in the main regions – from screening to *in product* approach

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Abstract

The increasing interest in products with functional properties has encouraged the search for new strains in natural sources. Traditional products, especially artisanal cheeses, have been the target of such studies because they present a wide microbial diversity, composed mainly of wild lactic acid bacteria (LAB) which stand out due to their singular metabolic characteristics resulting from resistance to food fermentation stresses. The aim of this study was to evaluate the probiotic potential properties of 220 *Lactobacillus* sp. strains that presented high enzymatic (proteolytic and lipolytic) and inhibitory activities against *Listeria monocytogenes* and *Staphylococcus aureus* in a previous study. These strains were isolated from samples of Brazilian artisanal cheeses produced in different regions. Resistance to low pH values and bile salts, hydrophobicity, auto aggregation assays, adhesion to Caco-2 cells and resistance to simulated conditions of gastrointestinal tract (GIT) were assessed. All strains were also submitted to PCR analysis in order to look up for virulent genes and verify its safety. In summary, 92 (41.81%) strains were resistant to at least one acid condition and submitted to bile salt tests and 22 strains showing high survival to 0.4% bile salt were submitted to adhesion assays. The values of auto aggregation (68.46 - 99.02%) were considered moderate/high and interesting for technological and therapeutic purposes due to their ability to prevent pathogenic adhesion to the intestinal mucosa. Hydrophobicity values varied greatly between strains (4.97 to 64.25%) and 7 strains presented values higher than 40%, indicating good potential for application as probiotics because hydrophobic cells exert an important role in the activation of the immune system of the intestinal mucosa, but it is not mandatory for adhesion. All strains were able to adhere to Caco-2 (> 70%) cells and 20 do not show virulent genes, being considered as safe. Most of the strains (88.81%) showed a good survival after passage through the simulated GIT. After this rational screening, one *Lactobacillus plantarum* (1QB77) isolated from artisan Minas cheese (from Serro microregion) were selected due its ability to produce bacteriocin-like antimicrobials able to inhibit the growth of pathogens *L. monocytogenes* and *S. aureus* in vitro. This strain was co-cultured *in product* using a microcheese matrix protocol with a mix of each pathogen mentioned and a reduction of approx. 4 log units were observed from the 7th day of ripening until submit ion to GIT stress (at day 21 of ripening). Even though the strains presented potential probiotic properties, before their commercial application, further studies are needed to assess their in vivo capability. Our results highlight that *Lb. plantarum* 1QB77 fulfills major in vitro probiotic criteria as well as interesting immunostimulatory properties, and thus may be a promising candidate for further in vivo studies aiming at the development of novel probiotic starter cultures with biopreservative and regional traits.

Keywords: Brazilian artisanal cheeses, endogenous microbiota, raw milk, probiotic potential, food safety, *Listeria monocytogenes*, *Staphylococcus aureus*.

1. Introduction

Latic bacteria (LAB) are commonly known to exhibit probiotic properties, representing the largest group in this regard. Among them, the *Lactobacillus* genus, together with *Bifidobacterium* and *Streptococcus* stand out, being intensely studied over the years, isolated from various food sources, mainly fermented and dairy-related products and considered as having a long history of safe use and are considered as GRAS (Arshad et al. 2018; Samedi and Linton Charles 2019; Yadav and Shukla 2017). Such foods are called as functional because in addition to their nutritional trait they also play benefits/aids to the host health by improving the intestinal microbial balance, which has been increasing its demand over the years due a more awareness population of a most natural and healthy diet to achieve well- being (George Kerry et al. 2018; Linares et al. 2017; Ruiz-Moyano et al. 2019). In addition, the increasing demand for new non-dairy functional products and the rise of vegetarianism has intensified searches for strains able to tolerate the harsh conditions applied during manufacturing of probiotic cultures and then to be artificially added to new formulations in viable and stable conditions (Colombo et al. 2018; Fiocco et al. 2019).

Artisanal cheeses stand out in this aspect as important reservoirs of wild LAB species, whose properties vary according to the region of isolation and type of processing due to differences in centesimal composition, salt concentration, oxygen levels, pH and moisture content of cheeses. These physicochemical features affect LAB metabolism, causing nuances in their stability and functionality at the time of consumption of the product. For the food to exert its probiotic appeal, it is known that the levels of LAB must reach a range of 10^8-10^9 cells in a daily intake of 100g of product (Colombo et al. 2018).

This requires that these microorganisms must meet certain selection criteria, dictated by FAO/WHO: to resist acid conditions of the gastrointestinal tract (GIT); adhere to intestinal mucosa and therefore colonize the colon; do not present virulence factors; be able to inhibit the growth of pathogens; resist stress during food processing (like acids, osmotic, suboptimal temperatures) and then come alive in the host's intestines and thus exert their benefits; be properly identified, characterized and qualified (Pimentel et al. 2017; Ruiz-Moyano et al. 2019; Yadav and Shukla 2017).

According to experts, in the functional product's market probiotic foods account for about 60-70% of the total, which has emerged an increasing interest from Brazilian food industries and investments in this trend, offering different alternatives to consumers. Moreover, several scientific studies involving artisanal products have been carried out in Brazil and worldwide in order to search for regional microorganisms with technological and probiotic potential aiming to obtain genetically-stable strains used as starters in food industry, to lower the costs in the production of dairy products and to value the application of regional dairy cultures, expanding Brazilian dairy industry (Colombo et al. 2018; Kamimura et al. 2019; Mahmoudi et al. 2018). These authors have found a diverse microbiota composition encompassing *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Weissella*, *Pediococcus* and *Leuconostoc* genera, strongly associated with the source of isolation and showing a great antagonist effect against pathogens (Martins et al. 2018; Campagnollo et al. 2018; Cavicchioli et al. 2017; Acurcio et al. 2017; Sant'Anna et al. 2017; Perin et al. 2017; dos Santos et al. 2014; Tulini, Winkelströter, and De Martinis 2013). From the point of view of public health, this ability to inhibit hazardous and spoiling microorganisms in food is one of the major concerns due to the recurrent outbreaks

associated with the ingestion of food-borne pathogens (Borges et al. 2009; Campagnollo et al. 2018; Mateus et al. 2017).

Thus, this study aims to continue the bioprospection of *Lactobacillus* sp. isolated from Brazilian artisanal cheeses produced in the main regions of the country and which have shown satisfactory results in a previous screening about their technological features (salt resistance, production of proteolytic and lipolytic enzymes, production of bacteriocin-like substances, exopolysaccharides). In the present study we evaluated the usual selection criteria for potential application as probiotics (low pH resistance, bile salts, GIT tolerance, adhesion properties) and a strain producing a bacteriocin-like substance able to inhibit species of *Listeria monocytogenes* and *S. aureus* was chosen to be applied in the production of microcheeses in order to verify its probiotic potential and its biosecurity against recurrent pathogens in a simulated artisanal microcheeses manufacturing.

2. Material and methods

2.1 Bacterial isolates

Lactobacilli strains were isolated in a previous study carried out by our laboratory to assess the diversity and technological aspects of lactic acid microbiota from 11 types of Brazilian artisanal cheeses (n=582) marketed in the five main regions of the country. All strains (n=220) able to produce extracellular enzymes (proteolytic and lipolytic), exopolysaccharides, diacetyl, biopreservative traits, among others features, were selected for this study. A total of 220 frozen stocks of LAB belonging to the *Lactobacillus* genera were routinely grown in de Man, Rogosa and Sharpe (MRS) (Merck,

Darmstadt, Germany) broth at 37 °C until exponential growth (~ 12 -16 h) before experiments.

2.2 Probiotic screening

Resistance tests to acid conditions, bile salts, adhesion assays (hidrophobicity, self-aggregation and in vitro adhesion to Caco-2 intestinal cells) and survival to the upper GIT were performed. In addition, in order to assess the safety of potentially probiotic LAB, they were also evaluated for carrying virulence genes.

2.2.1 Resistence to low pH and bile salts

For acid resistance tests, 1 % of each LAB culture grown overnight in MRS broth were inoculated into two modified MRS broth, whose pH was adjusted to 2 and 3 using an 1N HCL solution. Optical density at 600 nm (OD_{600nm}) was measured for 16 - 24 h in order to obtain growth curves under these conditions. Standard MRS broth was used as control. OD_{600nm} value of the final culture greater than 0.5 was considered as resistant to low pH (Bao et al. 2010). All strains able to resist acid conditions were subjected to bile salt stress. The test was performed according to Gotteland et al (2014). One percent of LAB cultures grown overnight in MRS broth were inoculated into MRS broth containing 0.4% bile salts (Oxgal) and incubated at 37 ° C for 4 h. LAB counts were carried out in MRS agar plates at 37 ºC for 48 h, using the microdrop plating technique. All strains whose initial concentration did not decrease by more than 2 log after incubation were considered as resistant. *Lactobacillus* sp. resistant to low pH and bile salts were selected for further evaluation about their adhesion properties and survival under GIT stress.

2.2.2 Adhesion screening

The specific cell–cell interactions were determined using the auto- aggregation assay described by Jeronymo-Ceneviva et al., (2014). LAB strains were grown in MRS broth at 30 ºC for 24 h. Cells were collected by centrifugation (7,000 g; 10 min; 20 ° C), washed, resuspended and diluted in sterile saline (0.85% NaCl). The absorbance of the suspension was then adjusted to 0.3 at 660_{nm}. One milliliter of the cell suspension was transferred to a 2 ml sterile microtube and incubated at 37 ° C for 1 h. The cells were then harvested by centrifugation (300 g; 2 min; 20 °C) and the final supernatant absorbance was measured again. Auto-aggregation value was calculated by the following equation: % Autoaggregation = $((A_0 - A_{60})/A_{60}) \times 100$. Where A_0 refers to the initial absorbance and A_{60} refers to the final absorbance, after incubation.

The cell surface hydrophobicity was performed according to Vinderola and Reinheimer (2003). LAB strains were cultured in MRS broth overnight and cells collected by centrifugation (8,000 g; 10 min; 5 ° C), washed twice and resuspended in phosphate buffered saline (PBS; pH 7.4)). The initial absorbance suspension was adjusted to 1.0 at 600_{nm}. Then, 3 mL of this suspension were added to 0.6 mL of a highly apolar reagent (n-hexadecane) (Sigma, St. Louis, USA), vortexed for 120 s and incubated at 37 ºC for 1 h. After incubation ended, it was possible to observe the separation of the polar and nonpolar phases. The aqueous phase was carefully removed for absorbance reading at 600_{nm}. The decrease in aqueous phase absorbance indicates cell surface hydrophobicity (% H), calculated by the following equation: $H\ \% = [(A_0 - A) / A_0] / 100$, where A_0 and A are the absorbances measured before and after extraction with n-hexadecane, respectively.

Adhesion to Enterocyte-like Cells (Caco-2) by LAB were performed according to Maragkoudakis et al (2006), with some modifications. Eukaryotic cells were routinely

grown in DMEM medium (Merck, Darmstadt, Germany) supplemented with fetal serum (10% w/v), 4 mM L-glutamine, 100 µg/mL penicillin and streptomycin (all from Merck, Darmstadt, Germany), non-essential amino acids (1% w/v) and amphotericin B at 0.50 µg/mL. Cells were incubated at 37 ° C under modified atmosphere containing 10 % CO_2. For adhesion tests, Caco-2 cell monolayers were prepared in 24-well tissue culture plates, seeded (1 mL per well) at a concentration of 1.0 x 10^5 cells/mL, adjusted with Neubauer chamber. The plates were incubated for 15 days (until complete differentiation) and culture medium changed every two days.

LAB cultures were grown in MRS broth (12 h, 10 mL, 37 ºC), collected (3,500 g, 10 min, 4 ° C) and washed twice with phosphate buffered saline (pH 7.2). The cells were then resuspended in 1 mL of the same buffer and diluted in non-supplemented DMEM medium to a concentration of 10^8 CFU/mL.

Growth medium (DMEM) was carefully aspirated from 24-well tissue culture plates containing Caco-2 cell monolayers (15 days old) and the eukaryotic cells were washed three times with PBS. Then 1 ml of the bacterial suspension in DMEM was deposited onto Caco-2 cell monolayer, in triplicate. The plates were then incubated at 37 ° C for 60 min under modified atmosphere containing 10% CO_2. After incubation time, bacterial cell suspension was aspirated from the plate and Caco-2 monolayers carefully washed (3 x) with PBS. After washing, 1 mL of 0.1% (v/v) Triton X-100 (Merck, Darmstadt, Germany) was added to each well in order to detach bacterial cells adhered to Caco-2 cells. The plate was further incubated for more 10 min at same conditions. The bacterial suspension was then enumerated by serial decimal dilution in PBS and surface seeding on MRS agar plates. The plates were incubated at 30 ° C for 48 hours. LAB adhesion data were expressed as the percentage of viable cells in relation to the initial concentration of

cells suspended in DMEM. The tests were performed in triplicate and *Escherichia coli* Dh5a and *Escherichia coli* ATCC 11229 strains were used as negative and positive controls, respectively.

2.2.3 Survival to simulated gastrointestinal tract (GIT) stress

The survival of LAB to gastrointestinal transit conditions was performed according to Burns et al (2010), with some modifications. The following components were used: i) simulated gastric juice (125 mM NaCl, 7 mM KCl, 45 mM NaHCO3 and 3 g / L pepsin) (Sigma, St. Louis, USA) with pH adjusted to 2.0 using a 0.1 N HCl solution; (ii) simulated duodenal juice containing 1% (w/v) bovine bile (Sigma, St. Louis, USA) at pH 8.0, adjusted with a 10 N NaOH solution and (iii) simulated ileum juice containing 0.3% (w/v) of bovine bile, 0.1% (w/v) of pancreatin (Sigma, St. Luis, USA) with pH 8.0 adjusted with a 10 N NaOH solution. Intestinally, 10 mL of LAB cultures grown in MRS broth for 24 h were collected by centrifugation at 10,000 g for 15 min at 5 ° C. Then the pellet was washed twice with physiological saline (0.85%) and the cells were resuspended in this same solution so that the concentration was adjusted to 10^8 CFU/mL. Then 1 mL of this suspension was centrifuged again, the supernatant was discarded and the pellet resuspended in 1 mL of simulated gastric solution and incubated at 37 ° C for 90 min with shaking (200 rpm), in order to mimic peristalsis. Subsequently, the cells were collected again (10,000 g, 10 min) and resuspended in duodenal juice and incubated for 10 min at 37 ° C. Finally, the cells were centrifuged, resuspended in simulated ileum juice and incubated for 90 min at 37 ° C. Viable cell counts were obtained before and after the simulation of each condition tested. The relative viability was calculated by comparing the colony-forming units (CFU) at initial (without stress) and final GIT-stressed bacterial

samples. Strains *Lactobacillus paracasei* PXN 37 (Protexin, Lopen Head, Reino Unido) and *Lactobacillus acidophilus* La-5 (Chr. Hansen, Hoersholm, Dinamarca) were used as positive controls.

2.3 Safety aspects

2.3.1 Genetic screening for virulence genes

In order to assess the safety of 22 LAB that showed good results in probiotic potential tests, PCR analyzes were performed to verify the presence of virulence genes (listed in Table 1.), according to Martín-platero et al. (2009), with modifications.

[insert Table 1 here]

2.3.2 DNA extraction

LAB strains were grown in MRS broth overnight (12 h, 30 ºC) and streaked onto MRS agar plates to obtain isolated colonies. After 48 h of incubation, one colony of each LAB was deposited into the bottom of a microtube (200 µL) and microwaved for 2 min at maximum power. Then 25 µl of DNA free water (Merck, Darmstadt, Germany) was added to each microtube and vortexed. DNA samples were frozen (- 80 ° C) and stored for further analysis. Prior to testing, a PCR reaction with a 16S universal primer was performed to confirm DNA quality.

2.3.3 PCR multiplex

PCR reactions were performed for a final volume of 25 µl containing 1x Taq reaction buffer, 2.5 mM MgCl2, 300 µM dNTPs, varying primer concentrations (as shown

in Table 2), 2.5 U taq polymerase (all purchased from Cellco, Sao Paulo, Brazil) and 2 µL of DNA. The temperature and time conditions of PCR reactions varied according to settled primer pairs (Martín-platero et al. 2009). The thermal cycler used for amplification was Veriti thermal cycler (Applied Biosystems, Paisley, UK). Amplicons were visualized in a UVB light transilluminator (Loccus Biotecnologia® - LTB-STi) after electrophoresis (150 V, 2h) on agarose gel, whose agarose concentrations varied according to the marker used (2.4 % for 100 bp; 1.8% for 200 bp and 1% for 1000 bp). The buffer used in the electrophoretic run was 1 x TAE (Sigma, USA) (40mM Tris, 40mM acetate, and 1mM EDTA, pH ~ 8.3). The gels were stained with 0.5 mg/mL ethidium bromide for 10 minutes and then captured using the L-Pix STi (Loccus Biotechnology®) photocumenter.

2.4 Inhibitory activity of Lactobacillus plantarum 1QB77 against pathogenic strains in a microscale cheese matrix

2.4.1 Pathogenic strains and preparation of cell suspensions

The strains used for this step were serious foodborne/spoilage causing bacteria*: L. monocytogenes* strain 3968 - serotype 1/2b isolated from cheese (LM 3968), *L. monocytogenes* strain 3973 - serotype 4b isolated from raw milk (LM 3973) and *Listeria monocytogenes* ATCC 7644; *Staphylococcus aureus* FRI 361, *Staphylococcus aureus* FRI S6 and *Staphylococcus aureus* ATCC 19095, all kindly donated by Oswaldo Cruz Foundation (Rio de Janeiro/RJ/Brazil) were used in order to obtain a mixed culture suspension for each pathogen to be applied in the microcheeses production. *L. monocytogenes* and *S. aureus* strains were cultured in tryptone soya broth added with yeast extract at 0,3 % (TSB, Oxoid, Basingstoke, UK) and Brain Heart Infusion broth (BHI,

Oxoid), respectively, and cell suspensions (10^8 CFU/mL) prepared according to Sant'Ana, Franco, and Schaffner (2012).

2.4.2 Semi-hard Minas microcheese production

Before each microcheese production, a suspension of *Lactobacillus plantarum* 1QB77 were prepared by inoculating it in MRS broth overnight (37 ºC, 16 h), washing 2 times with Buffer Phosphate solution (PBS) and adjusting the concentration using a McFarland scale (McFarland turbidimeter, MS Tecnopon, Piraci- caba/SP/Brazil) to 10^8 CFU/mL. Preparation of two mixed suspensions of *L. monocytogenes* (Mix 1=LM 3968, LM 3973, LM ATCC 7644) and enterotoxigenic *S. aureus* (Mix 2=SA FRI 361, SA S6, SA ATCC 19095) were performed as described above and the final cell concentration adjusted to 10^8 CFU/mL. Five treatments were done in duplicate on different days and consisted of the production of microcheeses added with: isolate 1QB77 alone, Mix 1 alone, Mix 2 alone, isolate 1QB77 co-inoculated with Mix 1 and 1QB77 co-inoculated with Mix 2. All microcheeses were assumed to contain naturally occurring microbiota. Additional LAB were added in a rate of 10^7 CFU/mL of milk, while the two mixes were added at 10^6 CFU/mL of milk to simulate massive contamination.

The laboratory-based production of semi-hard Minas microcheese was adapted from Campagnollo et al (2018) and Bachmann et al. (2009). Four hundred milliliters of pasteurized milk, purchased at a dairy farm (Araras/SP/Brazil), were warmed to 35 ± 1,00 ºC and added with 500 µL of $CaCl_2$ (saturated solution; 20 min), 360 µL of commercial rennet Estrella (85% bovine pepsin + 15% bovine chymosin, Chr. Hansen, Valinhos/SP/Brazil) and immediately distributed in 96 deepwell microplates. Each well already containg selected LAB and/or pathogen mix (one microplate per mix), depending

upon the treatment. After 40 min coagulation, curd cutting (using a 96-pin stirring device), slight agitation and resting for 30 min, the whey was drained by centrifugating microplates (4,800 g; 1 h; 30 ºC), descarting the supernant, and putting them upside down for dripping (30 min). Then, 11 µL of a sterile 36% (wt/vol) sodium choride solution was added to each well (to a final concentration of 2% salt in dry matter per cheese) and the plates centrifuged again. Finally, the plates were covered with a Breathseal (Sigma Aldrich, Germany), placed in a bag containg silica beads to adjust the moisture content of the microcheeses and ripen for 22 days at 22 ºC.

2.4.3 Microbiological analysis of semi-hard microcheeses

Microbiological analysis of semi-hard microcheeses during ripening included LAB, *L. monocytogenes* and *S. aureus* counting and was carried out according to Downes and Ito (2001) after manufacturing (day 0) and throughout ripening (days 7, 14 and 22). Each microcheese (~190 mg) were firstly 10 times diluted (first dilution) using 2% trisodium citrate (heated at 45 ºC) and then subbmited to serial dilutions. Viable numbers of LAB, *Listeria monocytogenes* and *Staphylococcus aureus* cells were determinated by plating appropriate dilutions in MRS, Oxford and Beard Parker agar, respectively. Also, cheese pH and moisture content were measured in non-pathogen inoculated cheeses using a portable pH meter coupled with knife electrode and temperature sensor (AK103 pHmeter, SC18 electrode, Akso Eletronic Products Ltda., São Leopoldo/RS/Brazil) and an eletronic Moisture meter (ID 200, Marte).

2.4.4 Microbiological analysis of semi-hard microcheeses

Considering the probiotic and bacteriocigenic properties of *Lactobacillus plantarum* 1QB_MRS_77, previously screened in Brazil (data not shown), all microcheeses were also subbmited to simulated GIT conditions in order to evaluate the survival of LAB and pathogens during GIT passage after ripening. The survival of LAB and pathogens to gastro-intestinal transit conditions was performed as described before (item 2.4.3), being N_{22} the number of cells on day 22 of ripening and the initial counts preceding GIT analysis. Growth potential (δ) of LAB 1QB77, *L. monocytogenes* and *S. aureus* strains in each treatment of GIT simulation (gastric, duodenal and ileum juice) performed to microcheeses was determined by calculating the difference between microbial counts (in logCFU/100 mg of cheese) after GIT stress (gastric (N_g), duodenal (N_d), ileum juices (N_i)) and the initial count (N_{22}). Each simulated juice were considered to support pathogen growth when δ was higher than 0.5 log CFU/100 mg of cheese (Jesus et al. 2016). When δ values were negative or lower than 0.5, each simulated juice was considered do not support pathogen growth.

2.5 Statistical Analysis

Quantitative data were expressed as mean ± standard deviation (SD) and submitted to one-way analysis of variance (ANOVA) to assess significant differences. Student's t-test was used, when necessary, to discriminate differences between means. The statistics software named PAST *version* 3.16 was used in these tests and a level of significance of 5 % ($p < 0.05$) was adopted.

3. Results and Discussion

3.1 pH and bile salts resistance

The present study aimed to evaluate the probiotic potential of 220 wild strains of *Lactobacillus* sp. previously isolated from 11 types of Brazilian artisanal cheeses and confirmed as having great biotechnological features – as extracellular enzyme, exopolysaccharide, bacteriocin like substances able to inhibit pathogenic strains, osmotic tolerance and diacetyl formation. First, all strains were submitted to low pH (2.0 and 3.0) stress, taking into account this is the first challenge during the passage through the host GT tract during digestion due to high gastric acidity, considered as the main obstacle to gut colonization (Fiocco et al. 2019). All strains showing growth (OD600nmf – OD600nmi > 0,5) at pH 2.0 and/or 3.0 in modified MRS were considered as resistant. After this first low pH resistance screening, 92 strains were chosen for the next step and submitted to bile salt resistance at 0.4 % (w/v) in modified MRS broth and LAB counts (logCFU/mL) were made before and after incubation for 4 h. From these counts it was possible to calculate the percentage of viable cells able to withstand bile salt stress.

[insert Table 2 here]

From all tested strains (n=220), 92 (41.82%) were resistant to at least one acidity condition, being 47.83 % isolated from Southeast-produced cheeses (Minas Artesanal Cheese), 27.17% from Northeast (Coalho and Butter), 17.39% from Central region (Caipira) and 7.61% from South (Serrano and Colonial). Most of these bacteria belonging to the species *Lactobacillus plantarum* (46.74%), followed by the species

Lactobacillus brevis (10.87%), *Lactobacillus paracasei* (9.78%), *Lactococcus lactis* (5.43%), *Lactobacillus rhamnosus* (2.17%), *Lactobaacillus paraplantarum* (1.09%), *Lactobacillus curvatus* (1.09%), *Lactococcus garviae* (1.09%) and *Pediococcus acidilactici* (1.09%).

Before reaching the intestinal tract and exerting its benefits to the hosts, potentially probiotic bacteria must withstand stress conditions during passage through the GIT, which involves low pH values and digestive enzymes present in the stomach. This pH value may vary from 1.3 to 4.5 during fasting or after a meal, respectively. (Handa and Sharma 2016; Pieniz et al. 2014). The species found in this study are in agreement with those found in studies conducted in Brazil and worldwide involving milk, cheese and traditional artisanal products and the higher occurrence of *Lactobacillus plantarum* also stands out in these matrices, due to its ubiquitous character, indicating that they are present in the environment (air, utensils), dairy and non-dairy products, fermented vegetables and human intestines (Abdali et al. 2018; Andrade et al. 2014; Arshad et al. 2018; Costa et al. 2013; Gheziel et al. 2019; Meira et al. 2012; Muhammad et al. 2019; N'tcha et al. 2016; de Sant'Anna et al. 2017). This tolerance of LAB to acid stress varies between species and is mediated by several protection mechanisms, such as F0F1-AT-Pase in the case of *Lactobacillus* sp. genus (Ruiz-Moyano et al. 2019).

In addition to acidic conditions, probiotic-potential LAB must also withstand the stress from bile salts present in the bile juice secreted by gallbladder in order to reside and colonize the small intestine of the host (Abosereh et al. 2016). The concentration of bile salts in the intestine may range from 0.1 to 0.5%, depending on diet and the amount of pancreatic juice secreted (Reis et al. 2016). Thus, regarding bile salts resistance only 22 isolates (23.91 %) showed survival rates above 75 % and 11 isolates (11.96 %)

between 50 and 75% (considered as moderate) when challenged to high concentrations of bile salts (0.4%). Most of them (59 isolates) had survival rates below 50%, representing 64.13% of the total. From the 22 strains selected to be more resistant to bile salts, the species of *Lactobacillus plantarum* (n = 14), *Lactobacillus paracasei* (n = 1), *Pediococcus acidilactici* (n = 1) and *Lactobacillus* strains identified only at genera level (n = 6) were highlighted. Most of these strains (n = 9) were isolated from Northeast region (from *Coalho* and *Manteiga* cheeses), followed by Southeast region (from Minas artisanal cheese), Central (Caipira cheese) and South (colonial cheese), being 8, 3 and 2 isolates, respectively.

These data are in agreement with those found by Silva et al (2019) when testing the bile salts tolerance of autochthonous LAB isolated from minas artisanal (Araxá microregion) obtained a small rate (30%) of resistant bacteria (mostly constituted of *Lb. plantarum* species) and a high variability intra-species, also encountered by other authors (Acurcio et al. 2014; Vinderola and Reinheimer 2003). These data indicate that the source of bacterial isolation may influence their resistance to bile salts. The sensitivity to bile salts derives mainly from their detergent action to bacterial cell membrane, consisting of lipids and fatty acids, and DNA (de Sant'Anna et al. 2017). On the other hand, the tolerance of LAB (mostly *Entrococcus* and *Lactobacillus* genus) to biliary salts is related to the production of biliary salts hydrolase (BSH) enzymes, which deconjugate biliary salts, decreasing their solubility and avoiding their detergent action. However, some LAB strains are not able to produce BSH and still survive in the gut, indicating that there are other tolerance mechanisms not yet elucidated (Vinderola and Reinheimer 2003). Among the benefits generated by LAB in the degradation of bile salts involved in the synthesis of cholesterol, we can highlight its decrease in the blood and the modulation of the ratio of

high-density lipoproteins and low-density lipoproteins (HDL-LDL), and consequently the reduction of hypertension cases (Samedi and Linton Charles 2019).

3.2 Survival through GIT passage

After initial screening, all 22 selected bacteria were subjected to an in vitro test simulating GIT passage and progressive stresses encountered during digestion (e.g., peristalsis, changes in pH, and changes in concentrations of enzymes and bile) (see item 2.2.4) in order to evaluate bacterial survival at the end of the process. All results are summarized in **Figure *1***.

[insert Figure 1 here]

Survival rates ranged from 64.01 to 101.74% for *Lactobacillus* sp. (1QB101) and *Lb. plantarum* (2QB431), isolated from Coalho and Caipira cheese, respectively. There was a great variability between species of *Lb. plantarum* and survival values of *Lactobacillus paracasei* 3QB321 (92.93%) were higher than commercial *Lb. paracasei* PXN 37 (83.21%). Strains of *Lb. plantarum* (2QB3953, QB397 and 2QB431) and *Pediococcus acidilactici* (2QB502) showed lower values than the positive control. These data are in agreement with studies carried out with autochthonous LAB strains isolated from Brazilian artisanal cheeses (70–100%), raw milk and artisanal ewes' cheeses (>98%), traditional fermented beverages (90.4%) whose values were above 70 %, demonstrating high GIT survival rates (reduction < 3 log CFU) and some of them even growing in the presence of artificial gastric juice (Costa et al. 2013; Handa and Sharma 2016; Pisano et al. 2014; dos Santos et al. 2015b; Silva et al. 2019).

The ability to tolerate low pH values found during GIT passage may be explained by the isolation source of these bacteria since the pH of Brazilian artisanal cheeses can reach values up to 4.7 (Kamimura et al 2019), which may favour the domain of well-adapted acid-tolerant bacteria. Additionally, LAB synthesize lactic acid from lactose, and probably need to be resistant to this acid to ensure their survival in acidic environments, i.e. cheese. According to N'tcha et al. (2016), there is a significant effect of acidification on the glycolytic activity of cells: under acidic conditions, the specific glucose consumption rate increased, enabling a large energy input allowing LAB to be more resistant to low pH and survive.

Since all LAB tested remained viable throughout GIT stress, which would allow their use as probiotics, all of them were subjected to adhesion tests in order to verify their colonization potential after passage through the GIT.

3.3 In vitro adhesion assays

Another key factor considered in the selection of potentially probiotic strains is their ability to adhere to epithelial cells and mucus present in the small intestine, which enhances probiotic intestinal colonization and extend residence time in the host, while preventing enteropathogen attachment and ensuring bacterial homeostasis in the intestinal flora. In order to assess adhesion capability, analysis of autoaggregation, hydrophobicity, and adhesion to Caco-2 cells (originally isolated from a human colon adenocarcinoma) were assessed and all data are summarized in **Table 3**, along with informations about their source of isolation.

[insert Table 3 here]

3.3.1 Autoaggregation

Bacterial aggregation between microorganisms of the same strain is of major importance for probiotics play their role in the host gut. In this study autoaggregation (Agg) values ranged from 68.46 to 99.02% for *Lactobacillus* sp. (1QB101) and *Lactobacillus plantarum* (1QB399), isolated from Coalho and Minas artisanal cheese (Canastra microregion), respectively. *Lactobacillus paracasei* (3QB321), isolated from Caipira cheese, and *Pediococcus acidilactici* (2QB502) strains, isolated from Colonial cheese, showed Agg values of 94.69 and 80.98%, respectively. It was observed that there was a great variability ($p<0.05$) between strains of *Lactobacillus plantarum*, depending on the isolation region. According to Rahman et al. (2008), all isolates were classified with high Agg potential (> 70%), except for isolate 1QB399, classified with medium Agg potential (20-70%). These results are in accordance to other studies involving *Lactobacillus plantarum* strains from bovine raw milk (~68.33%) and traditional fermented foods, as artisanal Siahmazgi white brined (~73.99%) and Coalho cheeses (~82.8%), brazilian ovine cheeses (~74.7%) and a fermented beverage of Himachal Pradesh (máx ~ 79.5%) (Gandomi et al. 2019; Handa and Sharma 2016; Mahmoudi et al. 2018; Meira et al. 2012; dos Santos et al. 2015b). The aggregation between bacterial cells is considered a major factor in adhesion and biofilm formation in order to achieve high cell density in the gut and then allowing attachment to various surfaces, such as intestinal mucosa and epithelial cells, forming a protecting barrier. Thus, cellular aggregation facilitates transient colonization contributing to the persistence of beneficial microorganisms in GIT and their health effects (Abbasiliasi, S. Tan, and Tengku 2017; de Melo Pereira et al. 2018; dos Santos et al. 2015b).

3.3.2 Hydrophobicity

The adhesion percentages of LAB to n-hexadecane are also shown in Table 3. Hydrophobicity values varied greatly between strains of the same species (*Lactobacillus plantarum*), ranging from 4.97 to 64.25% for strains 1QB77 and 2QB395, both isolated from Minas artisanal cheeses, but from different microregions: Serro and Canastra, respectively. This wide range of diversity between closely genetically related species and even between strains of the same species of probiotic LAB was also reported by other authors (Collado, Meriluoto, and Salminen 2008; Favaro et al. 2014; Ruiz-Moyano et al. 2019; dos Santos et al. 2015b). In a study performed by Vinderola et al. (2008), most intestinal *Lactobacillus* strains showed hydrophobicity values less than 40%, but four strains, including *Lb. gasseri* strains, had higher values, being classified with good hydrophobic potential. In our study, 7 bacteria of *Lactobacillus* sp. (1QB101), *Lb. plantarum* (2QB395, 2QB158, 3QB397, 3QB173, 3QB497) and *Lb. paracasei* (3QB321) have shown similar results. One strain of *Pediococcus acidilactici* (2QB502), isolated from Colonial cheese, showed low hydrophobicity (8,28%), which is in accordance with a low value (14.55%) encountered by Abbasiliasi et al. (2017) when testing *P. acidilactici* Kp10.

Hydrocarbon adhesion test has been widely used as a tool to measure the hydrophobicity of bacterial cell surfaces, mainly of lactobacillus and *Bifidobacteria* genus, due to its probiotic potential. These studies have shown that the presence of (glycol-) proteinaceous material at the cell surface results in higher hydrophobicity, which improves first contact between microorganisms and host cells, whereas hydrophilic surfaces are associated with the presence of polysaccharides. Although this property is a good indicator of adhesion, some studies have reported that it is not necessarily associated with good attachment to human cells (i.e. HT-29 cells) due to weak and easily reversible

bonds that just precedes the subsequent complex adhesion process mediated by more specific mechanisms involving cell-surface proteins and lipoteichoic acids. In conclusion hydrophobicity may aid adhesion, but is not a prerequisite for strong adhesion (Abbasiliasi, J. S. Tan, et al. 2017; Collado et al. 2008; Favaro et al. 2014; de Melo Pereira et al. 2018; dos Santos et al. 2015b; Todorov et al. 2008; Vinderola et al. 2007).

3.3.3 Adhesion to Caco-2 cells

Caco-2 cell adhesion is considered as the gold standard for in vitro evaluation of microbial adhesion. In this study, these values ranged from 71.15 to 81.00% for *Lactobacillus plantarum* strains isolated from Minas cheese from Canastra microregion (Medeiros, MG) and Campo das Vertentes microregion (Tiradentes , MG), respectively (**Table 3**). There was a significant difference between strains of the same species of *Lactobacillus plantarum*, but isolated from different types of cheese. All bacteria presented adhesion values above the negative control (62.89%) of *Escherichia coli* Dh5a (data not shown). These values were considerably higher than those found in other studies involving potentially probiotic lactic acid bacteria isolated from cheeses, kefir, traditional dairy foods (Gheziel et al. 2019; Leite et al. 2015; Maragkoudakis et al. 2006; Pino et al. 2019; Pisano et al. 2014; Uroić et al. 2014; Wang et al. 2010). Adhesion is the first defense mechanism against the invasion of pathogens and it is associated with the ability of LAB strains to establish links with molecules (receptors) of the internal intestinal surface and electrostatic and/or hydrogen bonds, preveting pathogenic colonization by steric interactions or specific blockage on cell receptors, providing a competitive advantage in the gastrointestinal system by secretion of antimicrobial substances, toxin inactivation, and hence immune stimulation. These properties have shown to exert a protective effect

against traveler's diarrhea, rotavirus diarrhea, and antibiotic associated diarrhea. (Collado, Meriluoto, and Salminen 2008; Mahmoudi et al. 2018; Samedi and Linton Charles 2019). In this study, there was a moderate positive correlation (0.56) among Agg and hydrophobicity values and a weak negative correlation (-0.21) among Agg and Caco-2 data. There was no correlation (-0.08) between Hydrophobicity values and adhesion to Caco-2 cells. These values are in agreement with studies involving LAB (Collado et al. 2008; Mahmoudi et al. 2018).

3.4 Virulent genes

Despite their general recognition as safe bacteria, the investigation of virulence factors in LAB with potential application in food products is relevant due to the risk of vertical gene transfer to intestinal pathogens or bacteria present in food systems, since these genes are usually located in transferable plasmids hence representing hazards for consumers (Martins et al. 2014). In order to complement the probiotic potential evaluation data of these 22 strains, results concerning the presence of genes associated with virulence factors (asa1, gelE, hyl, cyl, esp, efa and ace), production of biogenic amines (hdc1, tdc and odc), and vancomycin (vanA, vanB) resistance are shown in **Table 3**. Only 2 isolates of *Lactobacillus plantarum* (3QB_MRS_173 and 2QB_MRS_169), both from Coalho cheeses, were positive for gelE, asa1 and cyl, which are associated with factors involved in colonization or tissue invasion and hemolytic activity (Hendrickx et al. 2009). All isolates had the VanA gene (except isolate *Lactobacillus plantarum* 3QB_M17_397, from Canastra cheese), indicating a naturally resistance to vancomycin usually found in *Lactobacilli* sp. genus (Ruiz-Moyano et al. 2019) and none isolate had the VanB gene. No isolates showed genes encoding decarboxylase activity, which is associated with the

production of biogenic amines in fermented foods, representing a risk due its toxicity. Histamine, for example, is commonly found in cheese and other fermented dairy products and responsible for allergic reactions (dos Santos et al. 2015b). This indicates that of the 22 isolates of LAB with probiotic potential, 20 may be considered as safe candidates to be applied in lactic ferments of fermented foods.

3.5 Antipathogenic activity in microcheeses – an in product approach

One *Lactobacilli* strain was selected based on probiotic screening tests in vitro and assayed as adjunct culture in microcheese-making experiments at laboratory scale in order to asses its survival during ripening (~21 days) and its influence on pathogen growth *in product*. *Lactobacillus plantarum* isolate (1QB77) from Minas artisanal (Serro microregion) was selected due to its inhibitory capacity against *L. monocytogenes* and *S. aureus* by production of bacteriocin like substances (as verified in a previous study), besides the positive data evaluated in this study. **Figure *2*** shows viable cell counts of LAB, *S. aureus* and *L. monocytogenes* at days 0, 7, 14 and 22 of ripening in microcheeses made with and without (only the natural pasteurized raw milk microbiota) addition of LAB 1QB77 during manufacturing.

[insert **Figure *2*** here]

There was a reduction of 2.34 and 2.74 log units in *S. aureus* and *L. monocytogenes* cells after 21 days of ripening, respectively, in microcheeses inoculated with LAB 1QB77, with a decreasing rate of approx. 0.22 log units per day. In microcheeses manufactured without strain 1QB77 there was an increase in the population of both

pathogens of 1.45 and 1.29 log units, respectively. These data agree with Castro et al. (2018), who obtained an inhibition in the population of *S. aureus* of 2.82 log units in Coalho cheeses inoculated with endogenous *Enterococcus faecium*. Campagnollo et al (2018) obtained a decrease in about 4 log units of *L. monocytogenes* in semi-hard cheeses inoculated with endogenous LAB adjunct cultures isolated from Minas artisanal cheeses, which may be associated with the synergism between inoculated *Lb. plantarum*, *Enterococcus faecalis* and *Lb. brevis*, thus enhancing the bactericidal effect. Decay in pathogen concentrations indicates the presence of antimicrobial agents (such hydrogen peroxide, organic acids, diacetyl, carbon dioxide, and bacteriocins) synthesized by LAB during fermentation as a competitive advantage, thus acting as biopreservatives. There is a growing trend towards replacing artificial preservatives by natural ones, and Brazilian LAB represent a good alternative in this regard, increasing food safety and contributing to the terroir of regional foods, which sustain the Protected Appellations of Origin of local cheeses (Samedi and Linton Charles 2019).

Brazilian traditional cheeses have great historical and social importance, being part of people culture, promoting income for small producers. However, some are manufactured without proper facilities, good manufacturing practices, process standardization and many small handers employ raw milk, which decreases microbiological quality and increases the health risk of consumers due to the large manual contact present in the process. Thus, high concentrations of pathogenic strains involved in food outbreaks such as enterotoxin-producing *S. aureus* and the presence of *L. monocytogenes* are recurrent (kamimura et al, 2018; Resende et al., 2011; Castro et al., 2016; Campagnollo et al 2018; Castro et al, 2018; Silva et al, 2019). FSANZ (2009) have

found, for example, that 70% of all cheese implicated in foodborne illness outbreaks were produced with raw milk.

The occurrence of *L. monocytogenes* in semi-hard cheeses marketed in Brazil can vary from 1.4 to 6% (Raimundo et al 2013; Souza et al 2002; Silva et al, 1998), which is of major concern to the most vulnerable population composed of immunocompromised patients, pregnant women, new-borns and elderly, whose mortality rate reaches about 20 to 30% (FAO/WHO, 2002; McLauchlin, Mitchell, Smerdon, & Jewell, 2004); While the occurrence of *S. aureus* in artisanal cheeses is related to the occurrence of mastitis in herds and cheese contamination by handlers, who are carriers of these bacteria (Huber et al., 2010; Silva et al. 2019). Thus, the antagonistic activity of LAB is a fundamental probiotic property to take into consideration when selecting a start or adjunct cultures to be applied as biopreservatives in food formulations as an additional barrier to good manufacturing practices, mastitis control in dairy herds, contributing to food safety and functionality.

3.5.1 Growth potential (δ) pathogens after GIT stress

The main target site of probiotics is the human intestine, where they need to arrive as biologically active cells. In order to achieve and retain such amounts, probiotics need to survive the technological stress encountered during food manufacture, as well as the hostile environments found within the product and the host intestine. Some components of foods to which probiotics are added have been shown to be suitable for the maintenance and protection of strains from the exposure to low pH and bile salts during their passage through the gastrointestinal tract. Thus, cheese matrix is suitable for

the long-term survival of probiotic bacteria due its high buffering capacity and lipid content (Argyri et al. 2013; Reis et al, 2016; Fiocco et al, 2019).

Regarding the counts of *Lactobacillus plantarum* 1QB77 cells, there was an increase in the population until reaching about $5.5x10^8$ and $2.29x10^9$ CFU/100mg in microcheeses contaminated with *S. aureus* and *L. monocytogenes* (Fig 2), respectively. These data indicate the viability of these bacteria at high concentrations after stress processes found in cheeses (acidification, salting, redox potential), which is one of selecting criteria for candidate probiotic strains (Vasiljevic & Shah, 2008; Almeida Junior et al, 2015). Thus, in order to further verify the influence of this potentially probiotic strain on the growth of pathogens after progressive stresses found in the GIT passage, contaminated microcheeses was also subjected to the stresses described in item 2.4.4 and the growth potential of pathogens in each condition tested is shown in figure 3.

[insert Figure 3 here]

There was a reduction of about 3.93 and 4.42 log units in the counts of *S. aureus* and *L. monocytogenes* at the end of GIT passage in micro-cheeses inoculated with LAB 1QB77. In contrast, in uninoculated cheeses there was an increase in counts of 0.57 and 1.06 log units forth both pathogens, respectively. These data corroborate the probiotic activity of the selected strain, representing an additional hurdle for the pathogen population during the manufacturing of cheeses and after GIT stress. It is worth mentioning that a *S. aureus* population greater than 4 log CFU/g is necessary for the production of enterotoxins in milk and milk products. Thus, the success of LAB 1QB77 in the prevention of *L. monocytogenes* and *S. aureus* growth may help in the control of many

infections (i. e. acute infectious diarrhea, irritable bowel syndrome, necrotizing enterocolitis, ulcerative colitis and Crohn's disease) and protection of intestinal epithelium through a series of barriers and interference mechanisms, which is a remarkable feature. In addition, this ability can provide an alternative to the use of antibiotics in the treatment of these mentioned intestinal infections (Yadav et al 2017; Castro et al, 2018; Algostini et al 2018; Kings et al 2016).

As reported by others studies, *Lb. plantarum* represents an attractive biological agent with tremendous biotechnological potential, showing strong antagonism against a broad spectrum of foodborne pathogens, making this isolate (1QB77) desirable for exploring their potential for health benefits in the production of functional foods and, at the same time, interesting to design low-cost, probiotic starter cultures for developing countries like Brazil, considering the opportunity to use the same strain as food starter cultures and as probiotic starter cultures (Gheziel et al 2019; Handa et al, 2019; Behera et al 2018). In Brazil, despite the limitations and complexity of regulations about probiotics, this study points out artisanal cheeses as relevant sources of beneficial strains, enabling them to be further characterized (in vivo) to develop novel dairy products, with good acceptance by the consumers.

4. Conclusion

This study made it possible to investigate the probiotic potential of 220 LAB isolated from Brazilian artisanal cheeses and previously characterized as good technological strains. Twenty-two of them were selected as potential probiotic candidates due to their resistance to low pH values, bile salts and ability to adhere to the intestinal

mucosa. There were a great variability between same species of *Lb. plantarum* strains isolated from different cheeses, which points out the isolation region interferes in their performance. Based on this data, one strain of *Lactobacillus plantarum* (1QB77) was chosen, taking into consideration its ability to produce a bacteriocin-like antimicrobial. This strain has shown a great survival during cheese production, reaching high viable cell density (~10^9 logCFU/100mg of microcheese) at final ripening and reduced growth of *L. monocytogenes* and enterotoxigenic *S. aureus* populations in contaminated microcheeses at approx. 4 log units from the 7th day of ripening until *in vitro* GIT stress (at 21 day of ripening). Therefore, collection of LAB from spontaneously fermented and autochthonous products is of great significance, since artisanal products are still manufactured in a traditional way in households located in specific ecological spots and they can serve as a valuable source of microbiota containing LAB with new properties. This study aimed to address the lack of internationally published data on the bioprospecting of Brazilian autochthonous strains and proved to be effective in rational screening for probiotic strains, enabling production of functional cheeses with regional features and additional safety using biopreservatives isolated from the own artisanal cheeses, which improve quality without losing the tradition of Minas cheese. Additionally, adequate evidence of a general beneficial effect in humans is needed to confirm in vivo this finding.

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TABLES

Table 1. Sequência dos primers e produtos de PCR utilizados para a detecção dos genes de virulência e produção de aminas biogênicas.

Gene	Fator de virulência	Nome do primer	Sequência do oligonucleotídeo (5'-3')	Temepratura de anelamento (ºT)	Tamanho do produto (bp)	Referência
asa1	Aggregation substance	ASA 11 ASA 12	GCACGCTATTACGAACTATGA TAAGAAAGAACATCACCACGA	50	375	Vankerckhoven et al (2004)
gelE	Gelatinase	GEL 11 GEL 12	TATGACAATGCTTTTTGGGAT AGATGCACCCGAAATAATATA	47	213	
cylA	Cytolysin	CYT I CYT IIb	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT	52	688	
esp	Enterococcal surface protein	ESP 14F ESP 12R	AGATTTCATCTTTGATTCTTGG AATTGATTCTTTAGCATCTGG	47	510	
hyl	Hyaluronidase	HYL n1 HYL n2	ACAGAAGAGCTGCAGGAAATG GACTGACGTCCAAGTTTCCAA	53	276	
efaA	endocarditis antigen	EFA-AF EFA-AR	GCCAATTGGGACAGACCCTC CGCCTTCTGTTCCTTCTTTGGC	57	688	
ace	adhesion of collagen	ACE-F ACE-R	GAATTGAGCAAAAGTTCAATCG GTCTGTCTTTTCACTTGTTTC	48	1008	Martín-Platero et al. (2009)
vanA	vancomycin resistance	VAN-AF VAN-AR	TCTGCAATAGAGATAGCCGC GGAGTAGCTATCCCAGCATT	52	377	
vanB	vancomycin resistance	VAN-BF VAN-BR	GCTCCGCAGCCTGCATGGACA ACGATGCCGCCATCCTCCTGC	60	529	
hdc1	histidine decarboxylase	JV16HC JV17HC	AGATGGTATTGTTTCTTATG AGACCATACACCATAACCTT	46	367	
tdc	tyrosine decarboxylase	P2-for P1-rev	GAYATNATNGGNATNGGNYTNGAYCARG CCRTARTCNGGNATAGCRAARTCNGTRTG	55	924	De las Rivas et al. (2005)
odc	ornithine decarboxylase	3 16	GTNTTYAAYGCNGAYAARACNTAYTTYGT TACRCARAATACTCCNGGNGGRTANGG	54	1446	

Table 2. Number of LAB isolates (92 from a total of 220) tolerants to pH 2.0 and/or 3.0*, distributed per cheese type. LAB isolates showing great (75-100), moderate (50-75) and low/null (0-50) survival rates to bile salts (0.4 %) in modified MRS broth, distributed by cheese type.

LAB identity	Caipira				Araxá				Campo das Vertentes				Canastra				Serro				Coalho				Coalho light				Spicy coalho				Manteiga				Colonial				Serrano			
	pH 2/3*	0-50	51-75	75-100	pH 2/3	0-50	51-75	75-100	pH 2/3	0-50	51-75	75-100	pH 2/3	0-50	51-75	75-100	pH 2/3	0-50	51-75	75-100	pH 2/3	0-50	51-75	75-100	pH 2/3	0-50	51-75	75-100	pH 2/3	0-50	51-75	75-100	pH 2/3	0-50	51-75	75-100	pH 2/3	0-50	51-75	75-100	pH 2/3	0-50	51-75	75-100
Lactobacillus sp. (n=19)	3	3			6	5	1		4	2		2									2	1			1	1		1					2		1	1					1	1		
Lactobacillus plantarum (n=43)	7	4	1	2	2	1		1	5	3	2		8	3	2	3	2	1		1	10	4			5	2	1		1	1	1		1	1			3	1	1	1	2	2		
Lactobacillus brevis (n=10)	2	2							2	2							2	2			4	4																						
Lactobacillus paracasei (n=9)	2		1	1	2	2											4	4							1	1																		
Lactococcus lactis (n=5)	1		1										1	1			2	2					1										1		1									
Lactobacillus paraplantarum (n=1)													1	1																														
Lactobacillus curvatus (n=1)	1	1																																										
Lactococcus garviae (n=1)													1	1																														
Pediococcus acidilactici (n=1)																																					1			1				
Lactobacillus rhamnosus (n=2)													1	1			1	1																										
Total (n = 92)	16				10				11				12				11				16				4				1				4				4				3			

*calculated from the initial and final OD at 600 nm during incubation in modified MRS (pH adjusted to 2.5 and 3.5). Measurements above 0,5 were considered as able to resist to low pH conditions.

Table 3. NSLAB information about cheese type, region and microregion of origin (when applied) and their adhesion data: autoaggregation ability (AAg), surface hydrophobicity (H) and adhesion to human Caco-2 cells (Adh), in percentage. Results for virulence genes of potentially probiotic LAB obtained from Brazilian artinasal cheeses.

Strain code	LAB id	Cheese type	Region	Microregião	Aag (%)	H (%)	Adh (%)	*asa 1*	*gel E*	*cyl A*	*esp*	*hyl*	*efa A*	*ace*	*van A*	*van B*	*hdc 1*	*tdc*	*odc*
3QB321	*Lactobacillus paracasei*	Caipira	Central	-	94.69 ± 0.39^{cde}	62.81 ± 0.95^{abc}	73.94 ± 2.02^{abcdefghijk}	-	-	-	-	-	-	-	+	-	-	-	-
2QB431	*Lactobacillus plantarum*	Caipira	Central	-	87.46 ± 1.49^{ijkl}	16.19 ± 0.10^{m}	77.61 ± 2.11^{lmnopqr}	-	-	-	-	-	-	-	+	-	-	-	-
1QB350	*Lactobacillus plantarum*	Caipira	Central	-	91.37 ± 3.21^{efgh}	19.09 ± 2.31^{kl}	74.65 ± 0.88^{cdeghijklm}	-	-	-	-	-	-	-	+	-	-	-	-
3QB497	*Lactobacillus plantarum*	Colonial	South	-	96.60 ± 0.19^{abc}	42.10 ± 1.52^{g}	72.96 ± 1.92^{abcdefgh}	-	-	-	-	-	-	-	+	-	-	-	-
2QB123	*Lactobacillus plantarum*	Coalho	Northeast	-	88.84 ± 0.07^{ghij}	6.67 ± 1.73^{rstu}	71.77 ± 2.35^{abc}	-	-	-	-	-	-	-	+	-	-	-	-
3QB173	*Lactobacillus plantarum*	Coalho	Northeast	-	94.62 ± 1.45^{cdef}	52.28 ± 1.85^{ef}	78.61 ± 1.09^{pqrstu}	+	+	+	-	-	+	-	+	-	-	-	-
2QB158	*Lactobacillus plantarum*	Coalho	Northeast	-	80.67 ± 2.18^{nopq}	62.92 ± 0.57^{ab}	78.35 ± 1.78^{nopqrs}	-	-	-	-	-	-	-	+	-	-	-	-
3QB113	*Lactobacillus plantarum*	Coalho	Northeast	-	88.24 ± 0.73^{hijk}	20.17 ± 0.04^{k}	71.87 ± 1.35^{abcd}	-	-	-	-	-	-	-	+	-	-	-	-
2QB103	*Lactobacillus plantarum*	Coalho Light	Northeast	-	95.11 ± 2.14^{cd}	35.23 ± 0.02^{h}	72.34 ± 1.87^{abcde}	-	-	-	-	-	-	-	+	-	-	-	-
2QB169	*Lactobacillus plantarum*	Coalho	Northeast	-	92.35 ± 1.03^{defg}	33.46 ± 2.14^{hi}	78.52 ± 1.47^{nopqrst}	-	+	-	-	-	-	-	+	-	-	-	-
3QB66	*Lactobacillus plantarum*	Artisanal Minas	Southeast	Serro	69.04 ± 0.58^{tu}	8.86 ± 0.27^{qr}	77.34 ± 2.31^{lmnopq}	-	-	-	-	-	-	-	+	-	-	-	-
3QB216	*Lactobacillus plantarum*	Artisanal Minas	Southeast	Araxá	88.94 ± 1.63^{ghi}	14.67 ± 1.49^{mno}	75.56 ± 1.24^{ghijklmn}	-	-	-	-	-	-	-	+	-	-	-	-
2QB395	*Lactobacillus plantarum*	Artisanal Minas	Southeast	Canastra	98.74 ± 0.13^{ab}	64.25 ± 1.00^{a}	72.38 ± 3.16^{abcdef}	-	-	-	-	-	-	-	+	-	-	-	-
3QB397	*Lactobacillus plantarum*	Artisanal Minas	Southeast	Canastra	79.79 ± 2.01^{nopqr}	60.27 ± 0.39^{cd}	71.15 ± 1.57^{a}	-	-	-	-	-	-	-	-	-	-	-	-
1QB399	*Lactobacillus plantarum*	Artisanal Minas	Southeast	Canastra	68.46 ± 0.17^{tu}	7.14 ± 0.54^{qrst}	72.66 ± 1.61^{abcdefg}	-	-	-	-	-	-	-	+	-	-	-	-
1QB77	*Lactobacillus plantarum*	Artisanal Minas	Southeast	Serro	71.03 ± 1.89^{t}	4.97 ± 0.31^{tu}	76.09 ± 3.81^{lmnop}	-	-	-	-	-	-	-	+	-	-	-	-
1QB101	*Lactobacillus* sp.	Coalho	Northeast	-	99.02 ± 0.48^{a}	53.42 ± 1.29^{e}	73.42 ± 1.4^{abcdefghij}	-	-	-	-	-	-	-	+	-	-	-	-
1QB130	*Lactobacillus* sp.	Coalho Light	Northeast	-	81.31 ± 1.48^{no}	15.93 ± 0.27^{mn}	73.36 ± 0.65^{abcdefghi}	-	-	-	-	-	-	-	+	-	-	-	-
1QB177	*Lactobacillus* sp.	Manteiga	Northeast	-	82.04 ± 0.93^{n}	28.00 ± 0.50^{j}	74.51 ± 1.78^{cdeghijkl}	-	-	-	-	-	-	-	+	-	-	-	-
3QB287	*Lactobacillus* sp.	Artisanal Minas	Southeast	Campo das Vertentes	75.35 ± 0.24^{s}	12.25 ± 0.89^{op}	81 ± 2.02^{qrstu}	-	-	-	-	-	-	-	+	-	-	-	-
2QB288	*Lactobacillus* sp.	Artisanal Minas	Southeast	Campo das Vertentes	86.10 ± 3.17^{ijklm}	9.68 ± 0.89^{pq}	75.79 ± 2.47^{hijklmno}	-	-	-	-	-	-	-	+	-	-	-	-
2QB502	*Pediococcus acidilactici*	Colonial	South	-	80.98 ± 0.18^{nop}	8.28 ± 0.30^{qrs}	71.36 ± 2.09^{ab}	-	-	-	-	-	-	-	+	-	-	-	-

Means in the same column marked by different superscripts are significantly different ($p < 0.05$).

FIGURES

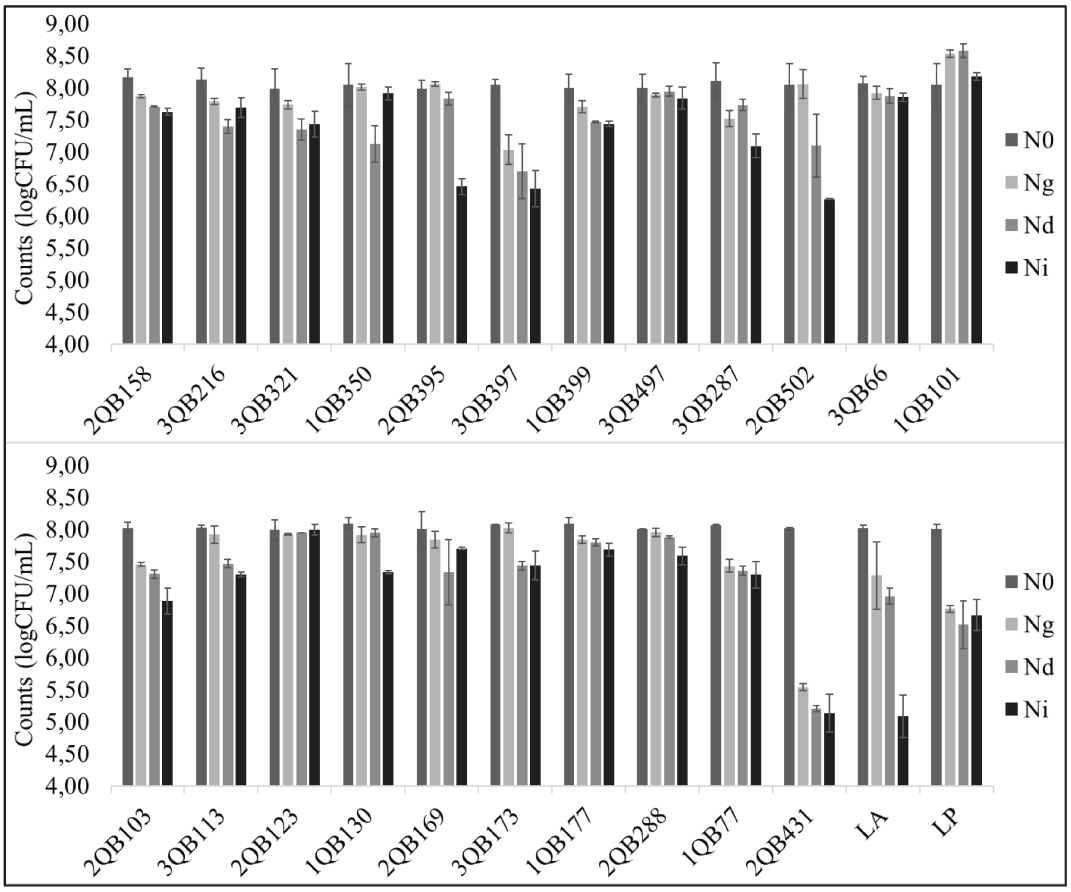

Figure 1. Survival test results for gastrointestinal transit (GIT), in logCFU/mL, where: N0: initial count, Ng: Count after treatment in simulated gastric juice; Nd: Count after treatment in simulated duodenal juice and Ni: Count after treatment in simulated ileum juice. The control strains were the commercial probiotic strains LA and LP, *Lactobacillus acidophilus* and *Lactobacillus paracasei*, respectively.

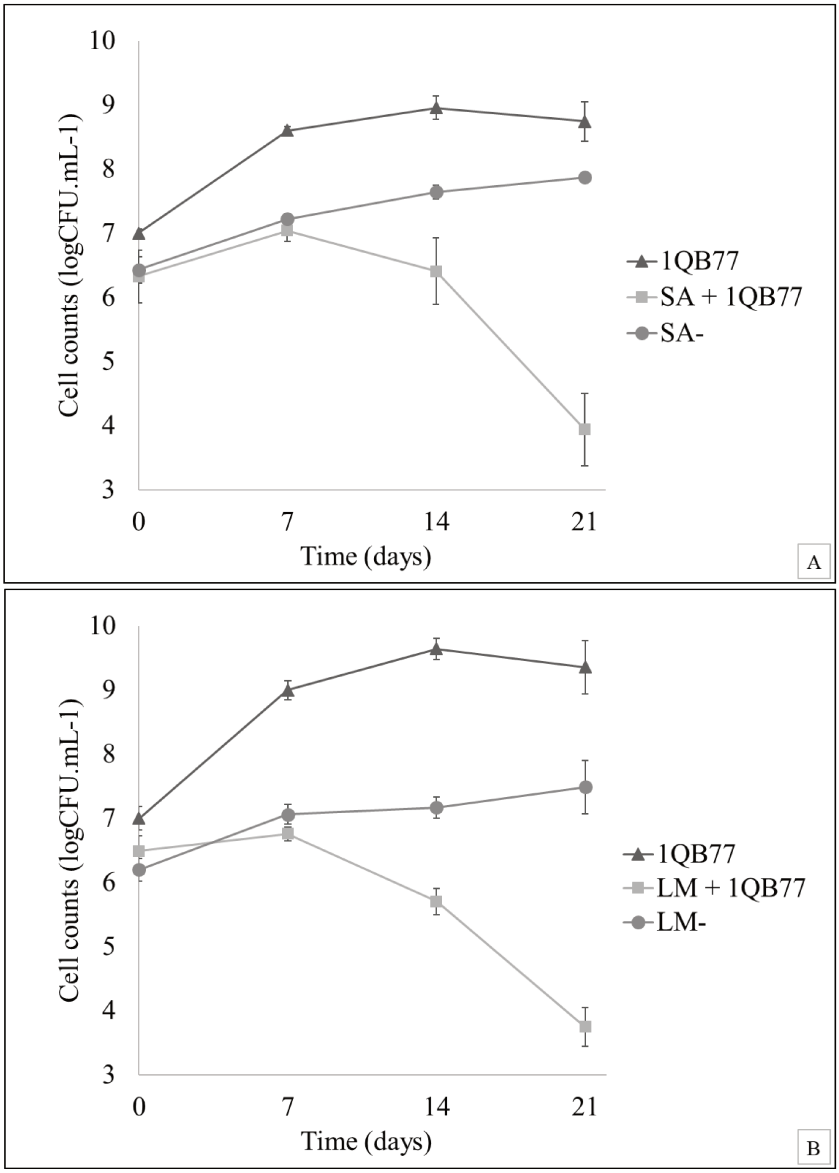

Figure 2. Counting of *Lactobacillus plantarum* (1QB77), *Staphylococcus aureus* (A) and *Listeria monocytogenes* (B) cells (in logCFU/mL) in micro-cheeses added with *Lactobacillus plantarum* 1QB77 plus pathogens (A: SA + 1QB77; B: LM + 1QB77) and without *Lb. plantarum* 1 QB77 (A: SA-; B: L-) per 0, 7, 14 and 21 days of ripening.

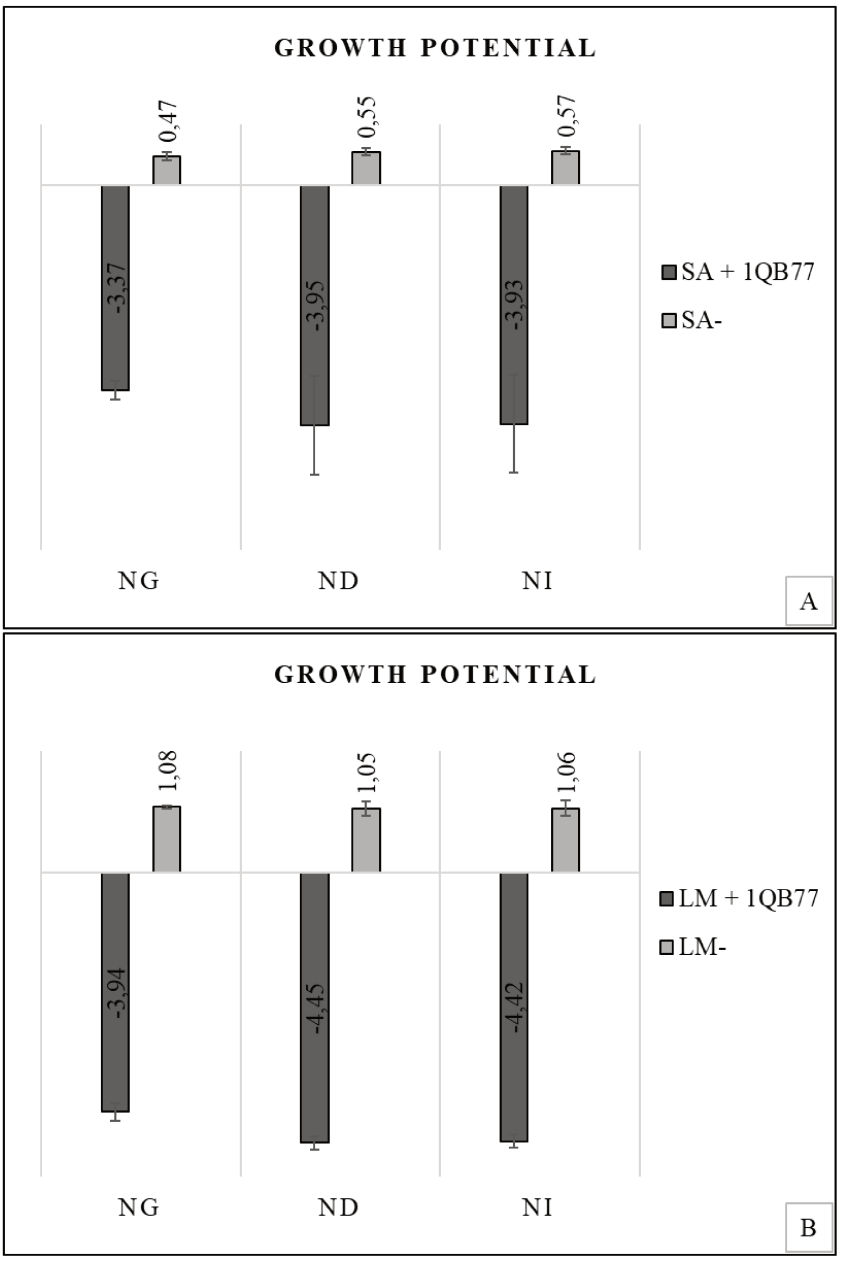

Figure 3. Growth potential (δ) of *Staphylococuus aureus* (SA) (A) and *Listeria monocytogenes* (LM) (B) in pathogen-inoculated micro-cheeses plus *Lactobacillus plantarum* 1QBMRS77 (SA + 1QB77; LM + 1QB77) and without *Lactobacillus plantarum* (SA) -; LM-) after 21 days of ripening followed by consecutively passage through simulated gastrointestinal transit conditions. “NG” = Counts after stress to simulated gastric juice; “ND” = Counts after stress to simulated duodenal juice; “NI” = Counts after debut to simulated ileum juice.

CAPÍTULO 5: Evolutionary engineering of a bacteriocigenic *Lactobacillus plantarum* isolated from Minas artisanal cheeses (Serro-MG) improves its probiotic features

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Evolutionary engineering of a bacteriocigenic *Lactobacillus plantarum* isolated from Minas artisanal cheeses (Serro-MG) improves its probiotic features

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Abstract

Lactobacillus plantarum species have a great versatility and beneficial properties, like the ability to inhibit foodborne pathogens, which is a desired feature in cheese production. In addition to the massively bioprospection studies involving traditional foods seeking for novel cultures, there is a more recent trend focused into improve the performance and efficiency of strain screening by new strategies, as laboratory evolution, also called evolutionary engineering. This technique aims the natural (non-Genetically modified) improvement of lineages and is based on the basic principle of genetic variation, which increases acceptance of consumers. Thus, this study aimed to apply laboratorial evolution in order to increase acidification rates in milk by successive transfers and tolerance to low pH (in initial acidification step present in cheese process) of an autochthonous bacteriocigenic strain isolated from Minas artisanal cheese (Serro-MG) who has shown good probiotic and biopreservative traits in a previous study reported by our group. After reaching up to 200 generations, it was observed that the evolved population of *Lb. plantarum* cells were six-times faster than the parental one, when acidifying milk. Representative colonies were picked up and applied in a microcheese matrix in order to compare *in product* evolved and parental strains during manufacturing, ripening and after a simulation though gastrointestinal tract (GIT) stress conditions. Evolved strains showed higher counts (1 log cycle) than the parental strains, meaning that 90% of the evolved *Lb. plantarum* population survived better than the parental ones to GIT stress. Parental and evolved strains did not present significant differences in physicochemical and aromatic properties of the micro-cheeses, which denotes evolution experiments as a useful tool to select probiotic strains capable of resisting acid stress without interfere in physicochemical features of microcheeses. Both evolved and parental strains were able to produce a great variety of aromatic compound currently present in most of cheese varieties, highlighting its potential to be applied as lactic ferments. In addition, due to the proximity of the values found in the microcheeses compared to the literature with pilot scale sample sizes (0.5–1kg), this protocol seems to be robust for future applications, in order to reduce the cost in screening tests for industrial applications (formulation changes, salt, addition of probiotics, among other aspects).

Keywords: Brazilian artisanal cheeses, lactic acid bacteria, *Lactobacillus plantarum*, evolutionary engineering, adjunct cultures, biotechnology, food quality, food safety.

1. Introduction

Brazilian artisanal cheeses play an important role in economy, social and cultural aspects and have been intensively studied due to its growing recognition as Cultural Patrimony of Brazil conferred by the National Historical and Artistic Heritage Institute (IPHAN) in 2006 because of its unique features mostly related to traditional and empirical techniques applied during manufacturing by small farmers, using raw milk and a diverse endogenous microbiota for over 200 years. This microbiota is composed mainly of lactic bacteria (LAB) and yeasts and plays a key role during ripening of these cheeses, improving flavor, colour and texture. It encompasses mainly the genera *Lactobacillus* sp., *Pediococcus* sp., *Enterococcus sp.* and *Lactococcus* sp. (Nóbrega, 2012; Borelli et al., 2006; Campagnollo et al., 2018; Kamimura et al., 2019; Kamimura et al., 2019).

Among the most recurrent isolates, *Lactobacillus plantarum* species stands out for its versatility and beneficial properties, being isolated from diverse ecosystems, mostly cheeses. Among these properties, we highlight the ability to inhibit a wide range of pathogenic microorganisms present in foods, which could contribute to safety and quality during their production (Rossi and Veneri, 2016; Behera, Ray and Zdolec, 2018; Gheziel et al., 2019). In general, raw-milk-produced cheeses should be ripened for over 60 days to be marketed in other states, according to actual brazilian legislation, which contrasts with the historical process performed in Minas Gerais at microregions of *Serra da Canastra*, *Serro* and *Salitre*, for example, which since the colonial period have ripened cheeses for about 20 days. Out of this, an aged period more than 60 days or pasteurization of milk should be applied, both negatively affecting its sensorial acceptance

due deterioration or presence of pathogens (Lima et al., 2008; Martins et al., 2015; Campagnollo et al., 2018).

In this context, the isolation of microorganisms naturally present in the microbiota of artisanal cheeses and the study of their biotechnological properties, such as lactic acid, enzymatic, flavor, aroma and antimicrobial activities has been increasing in order to obtain novel cultures with regional features (Ortolani et al., 2010; Tulini et al., 2016; Agostini et al., 2018). In contribution to these studies, a more recent trend has focused into improve the performance and efficiency of strain screening by new strategies based on random mutagenesis in combination with dedicated selection protocols to increase resistance of cells to the challenges present in fermentation processes i. e., strong acid, thermal and osmotic stresses, toxic products, and feedback inhibition, affecting its metabolic activity and hence reducing productivity. Thus, improving the stress tolerance of industrial strains is a critical factor in fermentation industry and should be also extended to traditional market as a new tool to increase safety and quality of Brazilian dairy products (Zhu et al., 2018).

The improvement of lineages through evolutionary engineering (or laboratory evolution) is based on the basic principle of genetic variation. In general, a population of cells is cultured under selection for many generations (cell divisions) and, using selective pressure as the driving force (Ukibe et al., 2009). Thus, random mutants will appear over time, usually after hundreds of generations, showing more relevant characteristics than their parental ones (Steensels et al., 2014). Evolutionary engineering mimics natural evolutionary processes, which consist of iterative cycles of genetic diversification and functional selection or screening, and unlike "rational" methods, is less dependent on prior knowledge of the genotype-phenotype relationship. Strains used in industrial processes

are often required to have several phenotypes, such as: tolerance to metabolic products, inhibitors and higher productivity, in order to meet the marketing criteria. Evolutionary engineering can be used for this purpose by the evolution and identification of adapted strains using selection criteria that reflect the process conditions, i.e. a selective pressure that acts as the driving force for the selection of mutants with improved phenotypes (Ukibe et al., 2009; Kim, Du and Zhao, 2013).

Due to short generation times and large population sizes, evolutionary adaptation of microorganisms to these experimentally imposed selection pressures can be easily studied in the laboratory (Barrick and Lenski, 2013). Moreover, another advantage of the application of this tool is that the strains obtained by evolutionary engineering are not considered as genetically modified, which increases the acceptance of the consumer market in Brazil. Evolutionary engineering is an extremely current technique and has been widely applied in biotechnological processes aimed at the production of biofuels, beer, milk and wine improving the performance of organisms like yeasts and lactic acid bacteria (Bachmann et al., 2012; Cadière et al., 2012; Ekberg et al., 2013; Tilloy, Ortiz-Julien and Dequin, 2014).

Considering the potential of the endogenous microbiota of artisanal cheeses for biotechnological application, such as the production of enzymes (proteolytic, lipolytic and glycolytic) essential for the ripening process of cheeses and resistance to processing conditions, this study aims to apply laboratorial evolution in order to increase acidification rates and tolerance to low pH of an autochthonous bacteriocigenic strain isolated from Artisanal Minas cheeses (Serro-MG) who has shown good probiotic and biopreservative traits *in product* in a previous study.

2. Material and methods

2.1 Pre-selection of LAB strains

A total of 9 LAB strains previously analyzed for their ability to inhibit growth of *Listeria monocytogenes* and enterotoxigenic *Staphylococcus aureus* strains, showing positive results in the bacteriocin tests (general features highlighted in Table 1) were picked up and evaluated regarding to their ability to acidify milk. The idea was to select one poorly acidifier strain but good bacteriocin-like substance producer and apply evolutionary engineering in order to increase their performance in the first stage of cheese production (acidification), and consequently their dominance of the microbiota. Thirty percent glycerol stocks of each strain were grown overnight in MRS broth at 37 ºC and inoculated in milk at 1% (v/v) in order to assess their acidifying ability.

[insert Table 1 here]

2.2 *Acidification rates*

The acidification assessment was made according to (Bachmann et al., 2013), with modifications. Cells were pre-cultured overnight in MRS broth at 37 ºC, two times washed in PBS buffer (pH 7.2) and the final OD adjusted to approximately 1 (~10^9 CFU/mL). Two microliters of the cell suspension were inoculated in 200 uL of milk containing 10 uM of -5-(6)-carboxyfluorescein (#21877, Sigma-Aldrich) in multiple wells of a black microplate with transparent bottom. Fluorescence emission at 520 nm (Excitation 485 nm) was measured in regular intervals of 1.5 hours in a microplate reader (Fluorostar, BMG LABTECH, City, Country). The acidification rates were measured based on the

decreasing fluorescence intensity of the pH-dependent indicator carboxy-fluorescein. All strains were considered as low acidifiers in milk but the fastest one (*Lactobacillus plantarum* strain 1QB77) was chosen and submitted to antagonism test against three starters of *Lactococcus lactis* (NCD 072, KF 147 and SK 11) in order to ensure it does not inhibit such frequently applied starters during cheese and vegetable fermentation processes.

2.3 Evolution experiments

The selected strain (1QB77) was experimentally evolved to perform better in milk (increased acidification rate) aiming to speed the processing of artisanal cheese according to Bachmann et al (2012a), with modifications. Cell suspensions were prepared as described previously and the initial OD_{600nm} adjusted to 1 (~ 10^9 CFU/mL). Then, 3 different tubes containing 10 mL of milk and a pH indicator purple bromocresol (80 mg/L) were inoculated with different initial cell concentrations (10^6, 10^7 and 10^8 CFU/mL) and incubated at 37 ºC until further coagulation (~ pH 5.0). After coagulation, a 10^4, 10^3 and 10^2-fold dilution was inoculated into 10 mL of milk again, separately. Successive transfers were performed, from each tube to another, until it reaches up to 200 generations calculated with the initial (N_0) and the final (N_f) population size after inoculation in a fully-grown culture ($N_f = N_0 \times 2^n$). These steps were performed in 3 replicates for each concentration (10^6, 10^7 and 10^8 CFU/mL).

2.4 Microcheese scale protocol for Artisanal Minas Cheese process – an in-product approach

The best evolved (with improved acidification rates) and parental strains were applied in the manufacturing of microcheeses, mimicking conditions of time, temperature for each step according to semi-hard Minas cheese production Before (Campagnollo, 2018), as described: standardized pasteurized raw milk (fat content of approximately 3.5%, protein content of approximately 3.4% a lactose content of 4.5%) from the pilot plant (Nizo Food Research) were warmed to 34 ºC for 30 min. Then, the following were added per 400 mL of cheese milk: 160 uL of $CaCl_2$ (0.4 $g.L^{-1}$, Akzo Nobel, Frankfurt, Germany) and 80 uL of rennet enzyme (kalase®, 150 IMCU, CSK Food Enrichment). Thereafter, 8 individual *Lb. plantarum* from the evolved and parental cultures were added to a final level of approximately 10^7 CFU/mL. All treatments were assumed to contain naturally occurring microbiota (e.g. indigenous LAB).

Following inoculation, the wells of a 2-mL deep-well microplate (Greiner, Alphen and Rijn, the Netherlands) were filled with 1.7 mL of milk each the plates were sealed with a capmat (Greiner) and incubated at 34 ºC using a MicroCheese model system developed by Nizo (patent number: PCT/NL2008/050369), as shown in Figure 1.B. The protocol for artisanal minas cheese was set up under the conditions shown in Figure 1.A, in order to mimic the steps during the pilot process of Minas Artisanal production (Figure 2).

[insert Figure 1 here]

[insert Figure 2 here]

2.5 Physicochemical and microbiological analysis of microcheeses

2.5.1 pH and Moisture

The pH of each microcheese was measured with a 3 mm electrode (BioTrode, Metrohm, Herisau, Switzerland) directly into the 96-deepwell microplate. The moisture content was determined using an electronic Moisture meter (Kern & Sohn GmbH, Balingen), by a gravimetric method.

2.5.2 LAB counting

Each microcheese (~ 190 mg) were homogenised using a stomacher and dissolved by adding 2% trisodium citrate (heated at 45 ºC) to obtain the first decimal dilution. Then, ten-fold serial dilutions were made in phosphate buffer solution and samples were plated on MRS agar and incubated at 37 ºC. The viable numbers of LAB during ripening were determinate in duplicate in the microcheeses after 0, 2, 7, 14 and 28 days.

2.5.3 Flavor formation

To determine the relative amount of aroma components in the micro cheese, a headspace solid phase micro extraction (SPME; Supelco, USA) gas chromatography coupled to mass spectrometry (HS-SPME-GC/MS; Fisons, USA) method was applied. Three individual microcheeses (~ 190 mg each) were put into a 10 mL amber screw cap vial. The solid phase extraction was carried out with a grey SPME fiber (Carboxen/PDMS/Divinylbenzene; Supleco, USA) for 20 minutes at 60 °C. Subsequently, the fiber was desorbed at 250°C. The extracted compounds were separated on a 30 m x 0.25 µm column with a VF-1ms (df = 1 µm) stationary phase (Varian, USA). The GC

separation started at 40°C for 1 minute, thereafter the temperature was raised with 10°C/min till 230°C. Mass spectra were recorded over a range of m/z 25-250.

2.5.4 Resistance to simulated gastric intestinal transit (GIT) conditions

Considering the probiotic properties of *Lactobacillus plantarum* 1QB77, previously screened in Brazil (data not shown), the microcheeses were also submitted to simulated gastric conditions in order to evaluate the resistance of the parental and evolved strains to the product process and gastric stress.

The survival of LAB (parental and evolved ones) to gastro-intestinal transit conditions was performed according to Burns et al (2010), with modifications. The following components were used: i) simulated gastric juice containing 125 mM NaCl, 7 mM KCl, 45 mM $NaHCO_3$ and 3 g/l pepsin (Sigma, St. Louis, USA) with pH adjusted to 2.0 using 0.1 N HCl solution; (ii) simulated duodenal juice containing 1% (w/v) bovine bile (Sigma, St. Louis, USA) at pH 8.0, adjusted with 10N NaOH solution and (iii) simulated ileum juice containing 0.3 % (w/v) bovine bile, 0.1 % (w/v) pancreatin (Sigma, St. Louis, USA) at pH 8.0 adjusted with 10N NaOH solution.

Then, each microcheese (~ 190 mg) were 10 times diluted (first dilution) using 2% trisodium citrate (heated at 45 ºC) and submitted to serial dilutions in order to obtain the initial courting (N_0) of LAB after 28 days of ripening and before the GIT stress. After that, 1.7 mL of the first dilution were submitted to centrifugation at 10,000 g for 15 min at 5 ° C in order to collect the pellets. The pellet was resuspended in 1.7 mL of simulated gastric solution and incubated at 37 ° C for 90 min under shaking (200 rpm). Subsequently, the cells were collected again (10,000 g, 10 min) and resuspended in duodenal juice and incubated for 10 min at 37 ° C. Finally, the cells were centrifuged, resuspended in

simulated ileum and incubated for 90 min at 37 ° C. Viable cell counts were obtained before and after the simulation of each condition tested, named as Ng (counting after gastric treatment), Nd (counting after duodenal treatment) and Ni (counting after ileum treatment).

3. Results and Discussion

3.1 Selection of LAB

Nine strains presented in Table 1 were selected in Brazil based on their bacteriocin activity against *Listeria monocytogenes* (LM 3968 and LM3973) and enterotoxigenic *Staphylococcus aureus* (FRI S6 and FRI 361), isolated from raw cheeses. The strains were identified as *Lactobacillus plantarum* (1QB314, 1QB352, 1QB371, 1QB77), *Lactobacillus brevis* (2QB422), *Lactococcus lactis* (1QB167), *Pediococcus acidilactici* (2QB502) and *Lactobacillus* sp. (1QB459, 3QB167, 1QB167) by MALDI-TOF MS analysis and submitted to acidification tests, as shown in Figure 3. It was noticed that all of them were considered as not good acidifiers and the strain 1QB77 (from Artisanal Minas cheeses from Serro) were selected due to their better performance compared to the others (it took 6 days to further coagulate the milk). Figure 4 also shows the data from antagonism tests performed against starters *Lactococcus lactis* SK11, KF 147 and NCD 072 from NIZO Food Research culture collection. None of tested strains inhibited the growth of *Lc. lactis*, widely applied in factory cheese and fermented products. This is interesting due to the role of starters in the early acidification step during cheese production and their possible application as mixed starter lactic culture together with the

bacteriocin producer *Lb. plantarum* 1QB77 regarding to safety aspects during cheese production.

[insert Figure 3 here]

[insert Figure 4 here]

Species of *Lactobacillus plantarum* are usually isolated from artisanal cheeses, being classified as adjunct cultures and contributing to the sensorial properties of ripened cheeses, several studies involving this bacterium has been developed, mostly reading to their probiotic potential and bacteriocin activity (Jeon et al., 2016; Zamani, 2016; Behera, Ray and Zdolec, 2018). Due to the accredited safety potency of bacteriocin origin and the wide-range effectiveness on pathogenic or spoilage bacteria, these compounds have attracted great research interest as natural antimicrobial agents, thereby allowing the design of new technologies for combating microbial pathogens in many industrial applications. In the case of artisanal cheese, the use of raw milk is hygienically hazardous and although good practices of milking and milk storage can reduce the risk of pathogen growth, it cannot nullify the risk completely. So, the possibility of exploiting the antimicrobial potential of bacteriocin, produced from LAB, to improve the safety of these products is a great alternative.

3.2 Laboratory evolution

Considering the increasing search for new alternative technologies to obtain more robust cultures with the aim of improving the production of cheeses with respect to safety and quality characteristics, an evolutionary engineering (EE) approach was used in order to select populations more resistant to the initial stage of acidification during cheese processing, enabling their dominance within the microflora. Also called as adaptative evolution, this technique has been used as one of the strategies to gain insight into the basic mechanisms of molecular evolution, resulting in improvements in the fitness and adaptive changes that accumulate in microbial populations during long-term selection under specific growth conditions, such as acid stress. The strain *Lb. plantarum* 1QB77 was then submitted to consecutive transfers in milk, in different initial inoculum concentrations, in 3 replicates, for 3 months. The aim was to increase the rates of acidification and bacteriocin production (which is growth-dependent) in milk. Figure 5 shows the evolutionary experimental design.

[insert Figure 5 here]

The use of different initial concentrations of inoculum was due to phenomenon called quorum sensing, which regulates some process in bacteria, like: development of genetic competence in *Bacillus subtilis* and *Streptococcus pneumoniae,* virulence development in *Staphylococcus aureus* and the production of antimicrobial peptides by several species of lactic acid bacteria. In the regulation mechanism of bacteriocin production, there are some induction factors (IF or pheromone) - bacteriocin-like peptides with 19-26 amino acid residues length, low molecular weight and cationic nature – that

play a great importance: the IF is constitutively produced and accumulated in low concentrations during bacterial growth. Then, induction of the bacteriocin genes occurs when IF concentration reaches the threshold for IF autoinduction level, so the control mechanism depends on the cell density of the cultures (Drider et al., 2006; Güllüce, Karadayı and Barış, 2013).

After reaching up to 200 generations (Table 2), it was observed that the population of *Lb. plantarum* cells present in tubes 2A, 10B and 10C (Fig 5b) started to acidify faster, with acidification rate dropping from 12 to 2 days, as shown in figure 6.

[insert Table 2 here]

[insert Figure 6 here]

The growth of LAB is characterized by the generation of acidic products of fermentation that accumulate in the extracellular environment. The pronounced organic acid production of these bacteria creates an environment unfavorable for many other organisms. This characteristic is the basis of numerous methods of food preservation by fermentation. In LAB one of the most effective mechanisms for resistance in acid stress environment is the glutamate decarboxylase (GAD), but they are also capable of inducing an Acid Tolerance Response (ATR) in response to mild acid treatments. Genes and proteins, involved in pH homeostasis and cell protection or repair, play a role in acid adaptation, but this role can also extend to more general acid tolerance mechanisms (van de Guchte et al., 2002).

The ability to decrease the pH were found in tubes 2a, 10b and 10c. Thus, five single colonies were selected from each tube and inoculated in MRS broth in order to perform acidification curves. The aim was to select 2 from each tube (with higher acidification rates) and apply them into a microscale cheese process in order to compare the parental and evolved isolates using an *in-product* approach. Thus, 8 strains were chosen for the microcheese production, being: strains 1 and 2, isolated from the parental stock culture (controls); 3 and 4, isolated from tube 2A (corresponding to transfer number 20ª during the EE with initial inoculum equivalent to 10^6 CFU/mL), 5 and 6, isolated from tube 10B (corresponding to transfer number 20ª during the EE with initial inoculum size equivalent to 10^7 CFU/mL) and strains 7 and 8, from tube 10C (corresponding to transfer number 23ª during EE with initial inoculum equivalent to 10^7 CFU/mL). Figure 7 present the microcheeses obtained by the Minas Artisanal cheese protocol described previously.

[insert Figure 7 here]

3.3 Microcheese analysis

Tables 3 and 4 show the pH and moisture values, respectively, throughout the microcheese processing after addition of renin (2.5 and 5.5 h), salting and over the course of ripening for 28 days. No significant differences were found in pH and moisture values between microcheeses inoculated with evolved and parental strains. However, the values obtained for each variable were within the range expected for Minas Artisanal cheeses (Pinto, 2008; Kamimura et al., 2019). Due to the non-standard process in the production of artisanal cheeses, there is a great variation in the pH and humidity values, influenced

by the season of the year in which the cheeses are produced, but the average composition of Minas cheese is 46–49 g/100 g moisture, 23–25 g/100 g fat, 20–22 g/100 g protein, 1.4–1.6 g/100 g salt with pH values ranging from 5.0 to 5.2 (Nogueira, Lubachevsky and Rankin, 2005). Due to the proximity of the values found in the microcheeses compared to the artisanal cheeses produced on a pilot scale, this protocol is robust for future applications, in order to reduce the cost of analysis aimed at the study of formulation changes, salt, addition of probiotics, among other aspects.

[insert Table 3 here]

[insert Table 4 here]

Considering that the wild-type strain of *Lactobacillus plantarum* 1QB77 also showed positive results in the screening of probiotic potential (performed in Brazil), microcheeses inoculated with parental and evolved strains were submitted to gastro-intestinal transit stress conditions, after 28 days of ripening. Thus, it was possible to evaluate the resistance of the strains to the processing steps (acidification, salting and ripening) and their survival to the gastrointestinal transit, concomitantly. Figure 8 shows the CFU counts of parental (strains 1 and 2) and evolved (strains 3 to 8) in microcheeses during ripening and after simulated GIT stress.

[insert Figure 8 here]

There is a slightly difference between the parental and evolved LAB counts after gastric treatment (pH ~ 2), with the evolved strains showing higher counts (around 1 log cycle) than the parental strains. It means that 90% of the evolved *Lb. plantarum* population survived better than the parental ones. Also, the counts of parental LAB were more unstable during the GIT stress simulation, showing a coefficient of variance of 5.58, 6.51 and 5.98 % in gastric, duodenal and ileum juices when compared to the evolved ones. Figure 8 summarizes the evolved and parental LAB counts and the changes in pH during each step, concomitantly.

The microcheeses inoculated with the parental (1+2) and evolved (3 to 8) strains were submitted to flavor analysis by gas chromatography. As we can see in the chromatogram shown in Figure 9 from the GC-MS analysis, 10 volatile compounds mainly including alcohols, aldehydes, ketones and organic acids were recovered from the microcheeses, being: acetic acid, diacetyl, acetoin, 1-butanol, acetone, 2-ethylpropanol, hexanoic acid, ethanol, 2-heptanone, and 2-propanol. The mean peak areas of each compound were calculated for the evolved and parental strains and summarized in Figure 10. There was no significant difference between the number of volatile compounds produced by the parental and evolved strains in the microcheeses tested.

[insert Figure 9 here]

[insert Figure 10 here]

Flavor development in dairy fermentations, most notably cheeses, results from a series of (bio)chemical processes in which the starter cultures provide the enzymes.

The major biochemical pathways which lead to the flavor formation of a whole range of precursors of flavor compounds are the following: metabolism of lactose, lactate, and citrate (often referred as glycolysis), proteolysis, and lipolysis. Primary products of these reactions may be further modified to a greater or lesser extent during ripening and lead to the formation of many volatile and non-volatile flavor compounds (McSweeney, 2004).

The strains of *Lb. plantarum* are considered heterofermentative, which means that lactic acid is the principal end product of fermentation but technologically significant amounts of one or more of the following metabolites are also produced, like: Carbon dioxide (CO_2) - which causes the small gas holes in Havarti, Gouda and other cheeses; Short chain fatty acids such as acetic acid and propionic; Acetaldehyde - a principal component of yogurt flavor; Diacetyl, a principal flavor note in sour cream, butter milk, Dutch cheese and Havarti cheese and Ethyl alcohol (Wegkamp et al., 2010).

The free fat acids (FFA) acetic acid and hexanoic acid produced originate probably from the lipolysis of the milk fat. The metabolic products of lactose catabolism, deamination of amino acids and possibly lipid oxidation may also had contributed to the overall FFA content. Due to their low aroma thresholds, short and medium-chain FFA are important contributors to the flavor profile in a wide variety of cheeses. Butanoic and hexanoic acids were also found in one study with Minas cheese, representing 40 and 25/100 g of the total FFA fraction, respectively, being characterized as having a strong, pungent cheese note (Nogueira, Lubachevsky, and Rankin 2005). In this study, acetic acid was the most abundant FFA encountered. This compound originates from a number of sources, including the metabolism of lactose by starter and nonstarter bacteria under cheese-like conditions, from citrate metabolism and amino acid catabolism and is characterized as having vinegar, pungent notes (Smid and Kleerebezem, 2014).

Ethanol, which is the main alcohol in many cheese varieties, is mostly produced through lactose fermentation and alanine catabolism and has been described as an important flavor component in a great diversity of cheeses, such as Cheddar, Feta and Serra da Estrela (Partidario, Barbosa, & Vilas Boas, 1998). Primary alcohols such as 1-propanol, 1-butanol, 1- pentanol, and 1-hexanol are produced mainly by the reduction of their corresponding aldehydes and methyl ketones. A possible contributor of fruity notes, 1-butanol was detected in significant quantities in this study, in agreement with Nogueira et al (2005) when analyzing Minas cheeses.

Methyl ketones like acetoin and 2-heptanone, have been important compounds in flavor of number of non-mold ripened cheeses such as Cheddar, Swiss Gruyere, Swiss cheese, Parmesan cheese and Grana Padano cheeses, being formed by enzymatic oxidative decarboxylation of fatty acids by the present lactic acid bacteria. Also, referend as 3-hydroxy-2-butanone, acetoin or acetyl methyl carbinol, can be also derived from diacetyl metabolism or from pyruvate metabolism during the conversion of lactose to lactic acid. Acetoin is characterized by buttery notes and was the third most found compound present in the microcheeses, which is in agreement with Nogueira et al (2005), who also found acetoin in Minas Cheese at concentrations above the aroma threshold of 1.0 mg/g.

4. Conclusion

Parental and evolved strains did not present significant differences in the physicochemical and aromatic properties of the micro-cheeses, although they achieved a significant reduction of the acidification rate (decreasing from 13 days to 2 days) in the milk after reaching 200 generations during the evolutionary engineering experiment.

However, a good difference in resistance to acidic pH of gastric juice between the evolved and parent strains (about 1 log cycle, which indicates a 90% greater viability) was observed. Thus, evolutionary engineering has proven to be a useful tool to select probiotic strains capable of resisting acid stress without interfere in physicochemical features of microcheeses. The wild and evolved strain of *Lb. plantarum* 1QB77 was able to produce a great variety of aromatic compounds currently present in most of cheese varieties. More studies should be performed in order to check the inhibition of pathogenic microorganisms in the microcheeses in order to corroborate the evolved strains application as probiotics and biopreservatives in cheeses, as already proved by the ancestral *Lb. plantarum* 1QB77.

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TABLES

Tabela 1. List containing 9 potential candidates for evolutionary experiments due to their bacteriocigenic activity against *Listeria monocytogenes* ATCC 3968 and ATCC 3973 (LM 3968 and LM 3973) and enterotoxigenic *Staphylococcus aureus* FRI S6 and FRI 361 (SA S6; SA 361).

LAB id	Bacteriocigenic potential					Cheese type	City	Region	Microregion
	Code	LM 3968	LM 3973	SA FRI S6	SA FRI 361				
Lactobacillu brevis	2QB422	+	+	-	-	Caipira	Ribas do Rio Pardo - MS	Mato Grosso do Sul	-
Lactobacillus plantarum	1QB314	+	+	+	+	Colonial	Lacerdópolis - SC	Sul	-
Lactobacillus plantarum	1QB352	+	-	-	-	Minas artesanal	São João del Rei - MG	Minas Gerais	Campo das Vertentes
Lactobacillus plantarum	1QB371	+	-	-	-	Minas artesanal	São João del Rei - MG	Minas Gerais	Campo das Vertentes
Lactobacillus plantarum	1QB77	+	+	+	-	Minas artesanal	Serro - MG	Minas Gerais	Serro
Lactobacillus sp.	1QB459	+	+	+	-	Caipira	Jaraguari - MS	Mato Grosso do Sul	-
Lactobacillus sp.	3QB167	+	+	+	-	Manteiga	Cajazeiras - PB	Nordeste	-
Lactococcus lactis	1QB167	+	+	+	-	Manteiga	Cajazeiras - PB	Nordeste	-
Pediococcus acidilactici	2QB502	+	+	+	-	Colonial	Carlos Barbosa - RS	Sul	-

Table 2. Describes the number of transfers carried out along the evolutionary engineering and the calculation of the number of generations according to the initial concentration of inoculum (No) per tube, in triplicate (A, B, C). The number of generations were calculated using the following equation: n = (logN-logNo)/log2, where: N = final population, No = initial population. Time indicates the number of days to full acidification (pH<5.1).

Transfers	No = 2x10^5						No = 2x10^6						No = 2x10^7					
	2A		2B		2C		10A		10B		10C		100A		100B		100C	
	Time	n	Time	n	Time	n	Time	n	Time	n	Time	n	Time	n	Time	n	Time	n
1	5.00	13.29	5.00	13.29	5.00	13.29	5.00	9.97	5.00	9.97	5.00	9.97	5.00	6.64	5.00	6.64	5.00	6.64
2	9.00	13.29	9.00	13.29	9.00	13.29	9.00	9.97	9.00	9.97	9.00	9.97	9.00	6.64	9.00	6.64	9.00	6.64
3	12.00	13.29	12.00	13.29	12.00	13.29	12.00	9.97	12.00	9.97	12.00	9.97	12.00	6.64	12.00	6.64	12.00	6.64
4	13.00	13.29	13.00	13.29	13.00	13.29	13.00	9.97	13.00	9.97	13.00	9.97	13.00	6.64	13.00	6.64	13.00	6.64
5	11.00	13.29	11.00	13.29	11.00	13.29	11.00	9.97	11.00	9.97	11.00	9.97	11.00	6.64	11.00	6.64	11.00	6.64
6	5.00	13.29	10.00	13.29	10.00	13.29	10.00	9.97	5.00	9.97	4.00	9.97	13.00	6.64	13.00	6.64	10.00	6.64
7	5.00	13.29	9.00	13.29	9.00	13.29	9.00	9.97	5.00	9.97	4.00	9.97	12.00	6.64	12.00	6.64	9.00	6.64
8	5.00	13.29	9.00	13.29	9.00	13.29	9.00	9.97	3.00	9.97	3.00	9.97	13.00	6.64	13.00	6.64	10.00	6.64
9	3.00	13.29	10.00	13.29	10.00	13.29	10.00	9.97	4.00	9.97	3.00	9.97	-	-	-	-	-	-
10	3.00	13.29	-	-	-	-	-	-	3.00	9.97	3.00	9.97	-	-	-	-	-	-
11	4.00	13.29	-	-	-	-	-	-	4.00	9.97	2.00	9.97	-	-	-	-	-	-
12	3.00	13.29	-	-	-	-	-	-	3.00	9.97	2.00	9.97	-	-	-	-	-	-
13	2.00	13.29	-	-	-	-	-	-	3.00	9.97	3.00	9.97	-	-	-	-	-	-
14	2.00	13.29	-	-	-	-	-	-	2.00	9.97	2.00	9.97	-	-	-	-	-	-
15	2.00	13.29	-	-	-	-	-	-	2.00	9.97	2.00	9.97	-	-	-	-	-	-
16	2.00	13.29	-	-	-	-	-	-	2.00	9.97	2.00	9.97	-	-	-	-	-	-
17	2.00	13.29	-	-	-	-	-	-	2.00	9.97	2.00	9.97	-	-	-	-	-	-
18	2.00	13.29	-	-	-	-	-	-	2.00	9.97	2.00	9.97	-	-	-	-	-	-
19	2.00	13.29	-	-	-	-	-	-	2.00	9.97	2.00	9.97	-	-	-	-	-	-
20	2.00	13.29	-	-	-	-	-	-	2.00	9.97	2.00	9.97	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-	2.00	9.97	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-	2.00	9.97	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-	2.00	9.97	-	-	-	-	-	-
Generations	265.75		119.59		119.59		89.69		199.32		229.21		53.15		53.15		53.15	

Table 3. Average pH of microcheeses inoculated with evolved and parental strains of *Lb. plantarum* 1_QB_MRS_77 during 28 days of ripening.

	pH						
Strains	2.5 h (after pressing)	5.5 h	1 day (unsalted)	2 days (with salting)	7 days	14 days	28 days
parental (1+2)	6.19	5.86	5.51	5.26	4.94	4.72	4.97
evolved (3+4)	6.19	5.86	5.49	5.27	4.93	4.78	5.02
evolved (5+6)	6.21	5.86	5.51	5.27	4.89	4.71	4.87
evolved (7+8)	6.28	5.89	5.49	5.29	4.88	4.74	4.97

Table 4. Average moisture content (in %) of microcheeses inoculated with evolved and parental strains of *Lb. plantarum* 1_QB_MRS_77 during 28 days of ripening.

	Moisture (%)						
Strains	2.5 h (after pressing)	5.5 h	1 day (unsalted)	2 days (with salting)	7 days	14 days	28 days
parental (1+2)	-	50.95	48.08	49.41	-	-	45.26
evolved (3+4)	-	47.98	46.35	45.94	-	-	45.02
evolved (5+6)	-	49.53	46.04	46.82	-	-	45.54
evolved (7+8)	-	50.47	45.41	47.39	-	-	45.64

FIGURES

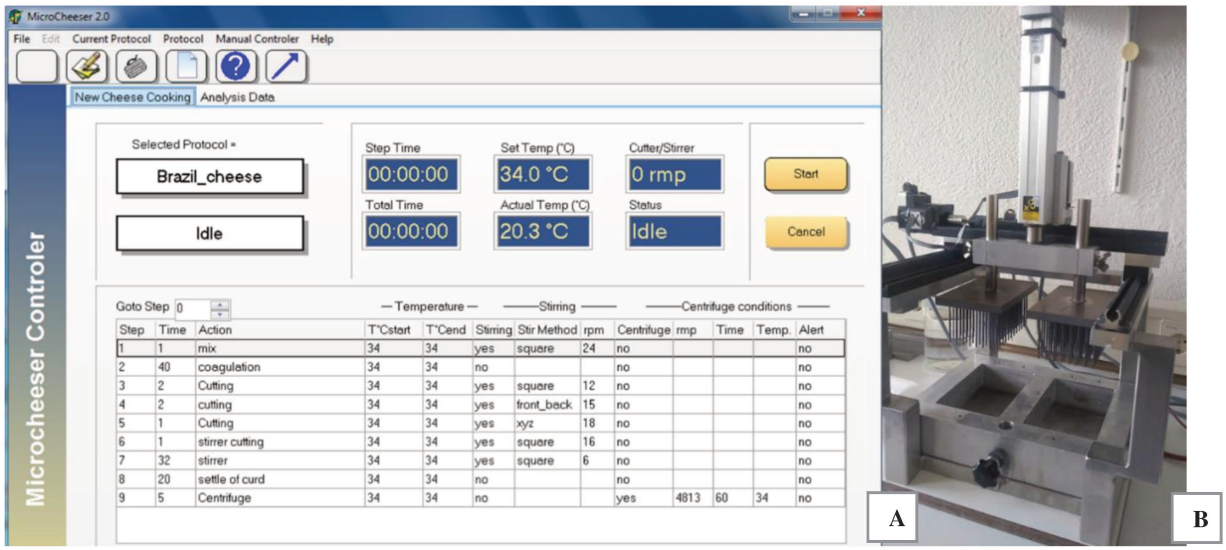

Figure 1. Minas Artisanal microcheese protocol (A) setted up using the Microcheeser 2.0 (B) developed by Nizo Food Research.

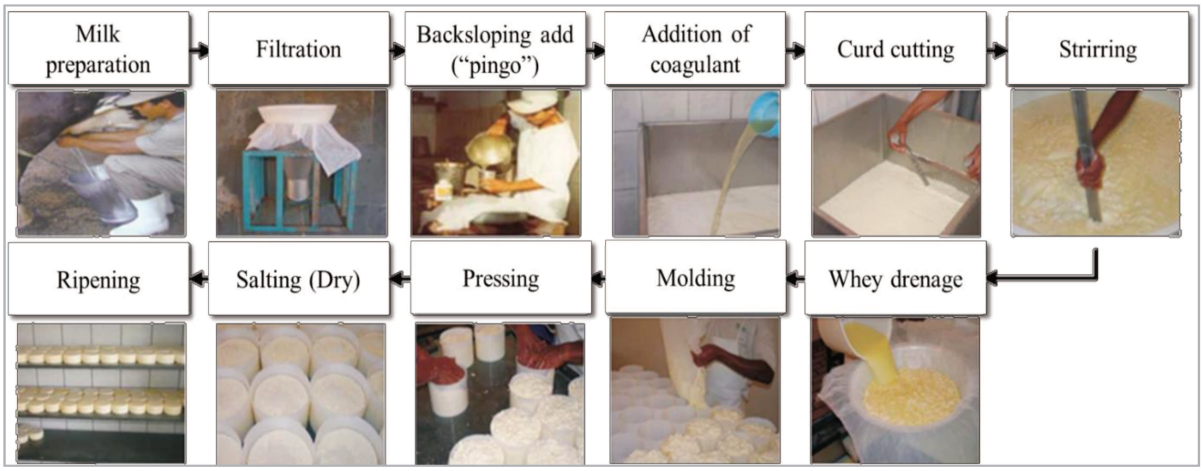

Figure 2. Minas Artisanal Process of cheese.

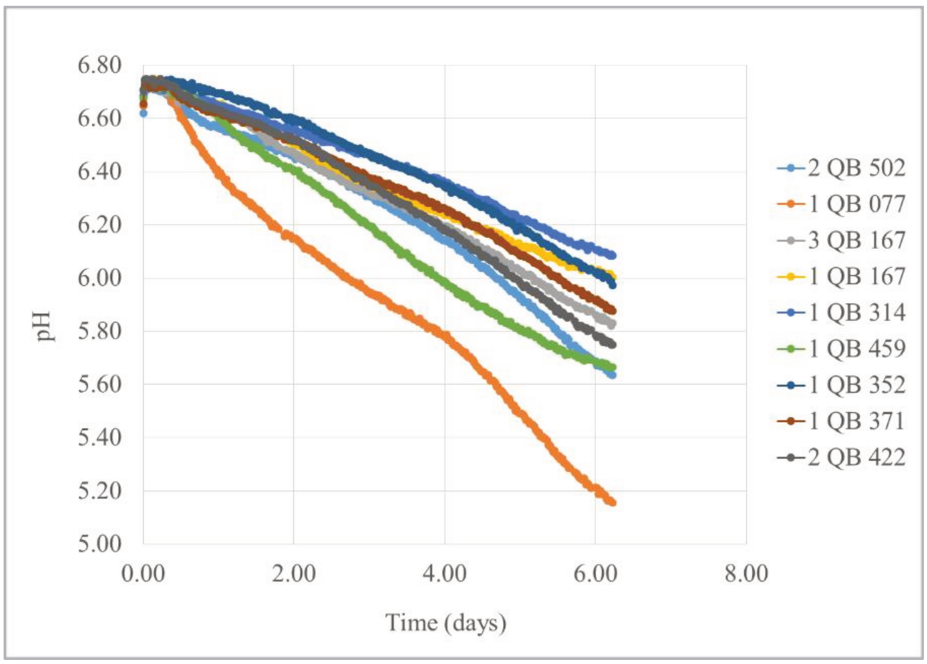

Figure 3. Acidification profile (pH vs days) of nine bacteriocin producer species of LAB isolated from brazilian artisanal cheeses (features shown in Table 1).

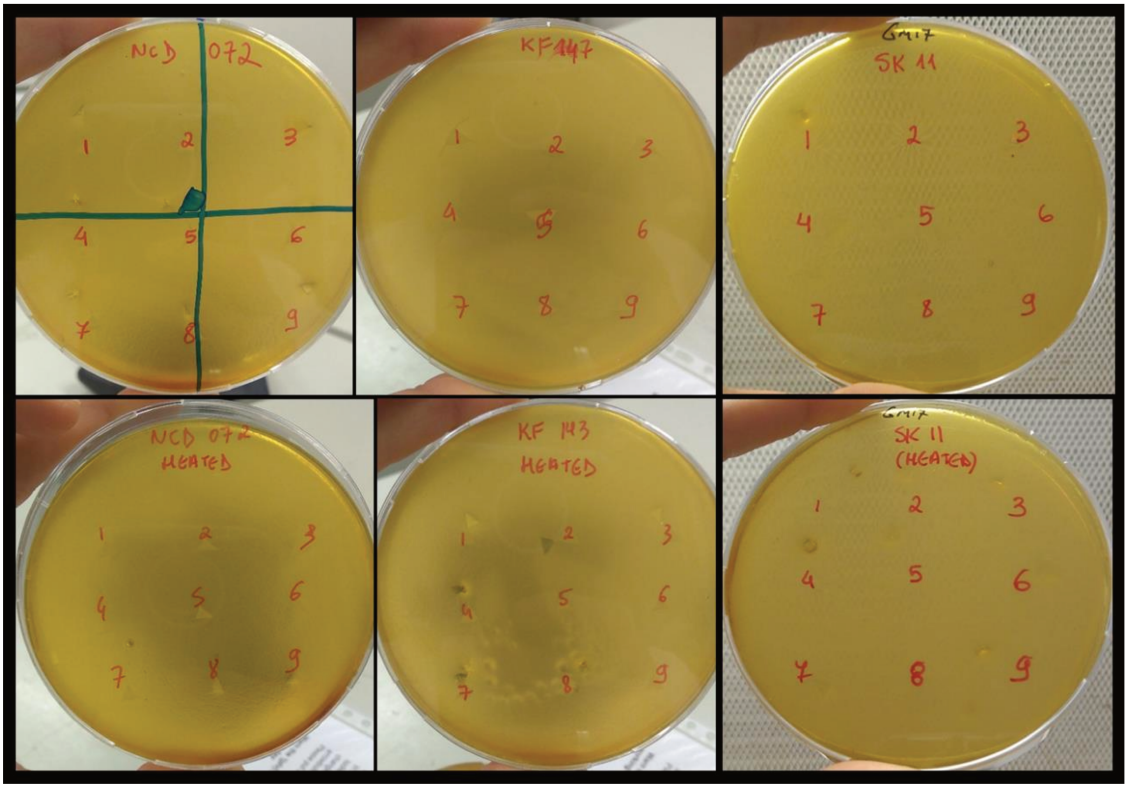

Figure 4. Antagonic test of 9 wild *Lb. plantarum* (listed from 1 to 9) against *Lactococcus lactis* species SK11, KF 147 and NCD 072, current applied in cheese production. The absence of halos indicated no inhibition of the starters by the *Lb. plantarum* tested.

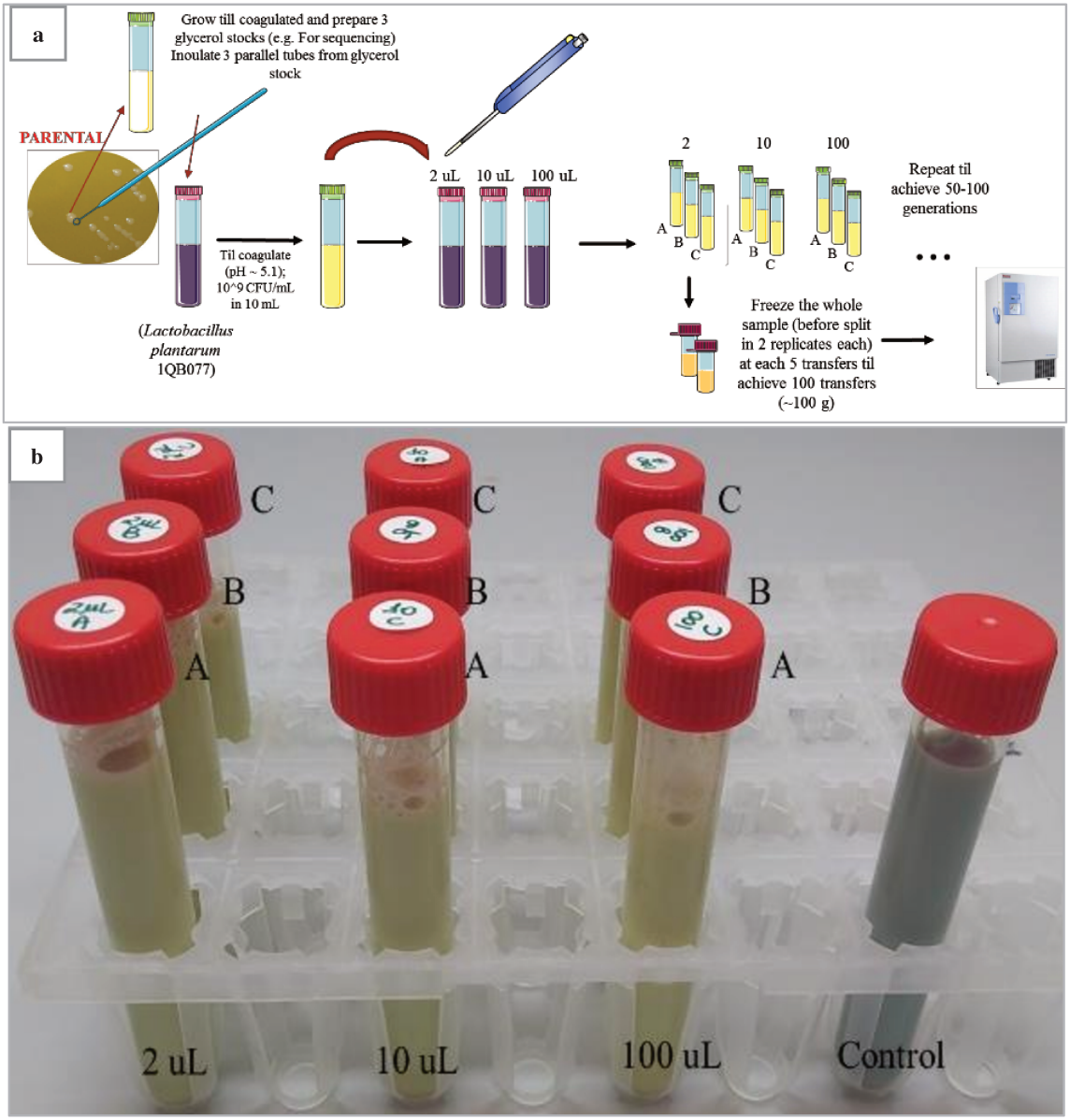

Figure 5. Evolutionary experiment set up (a). Tubes containing different inoculums size of *Lb. plantarum* 1QB77. The tubes were named as 2A, 2B, 2C (replicates of 2 uL inoculated in 10 mL of milk, reaching a initial inoculum size (~ 10^6 CFU/mL), 10A, 10B, 10C (replicates of 10 uL inoculated in 10 mL of milk, reaching a initial inoculum size around 10^7 CFU/mL) and 100A, 100B and 100C (replicates of 100 uL inoculated in 10 mL of milk, reaching a initial inoculum size about 10^8 CFU/mL) (b).

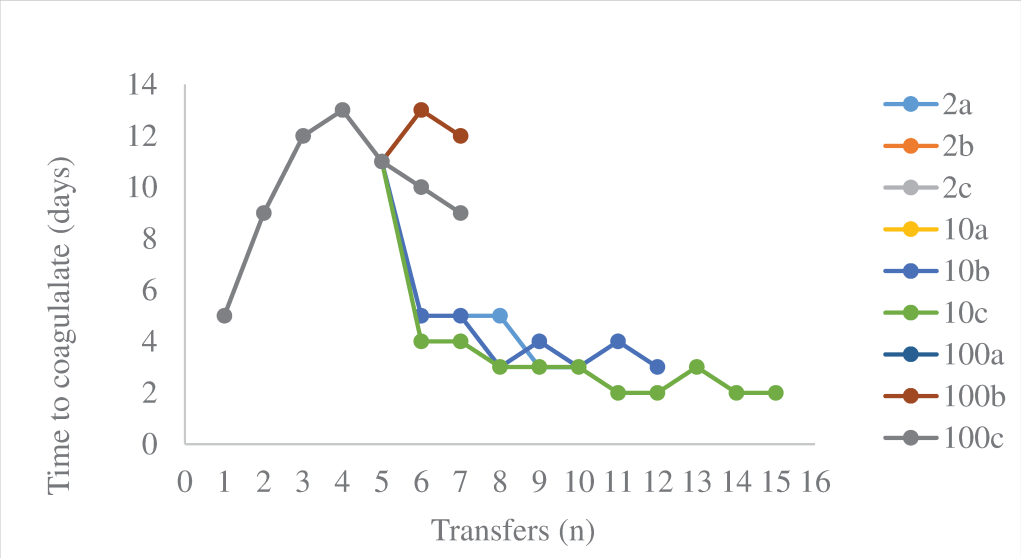

Figure 6. Number of days necessary to decrease the pH of milk until coagulation during the propagation steps (transfers). 2a, 2b, 2c: inoculation at 0.001% (2uL per 10 mL of milk); 10a, 10b, 10c: inoculation at 0.01% (10 uL per 10 mL of milk); 100a, 100b, 100c: inoculation at 0.1% (100 uL per 10 mL of milk).

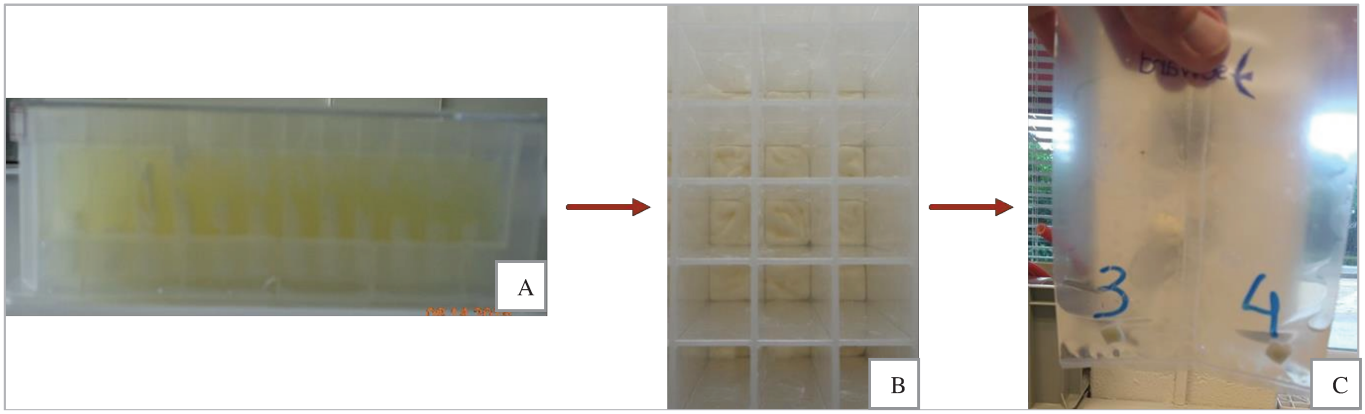

Figure 7. Pictures of the microcheeses obtained by the Minas Artisanal cheese protocol. Deep-well microplate before whey remotion by centrifugation (A); Microcheeses after centrifugation (B) and diluted in citrate sodium for CFU counting (C).

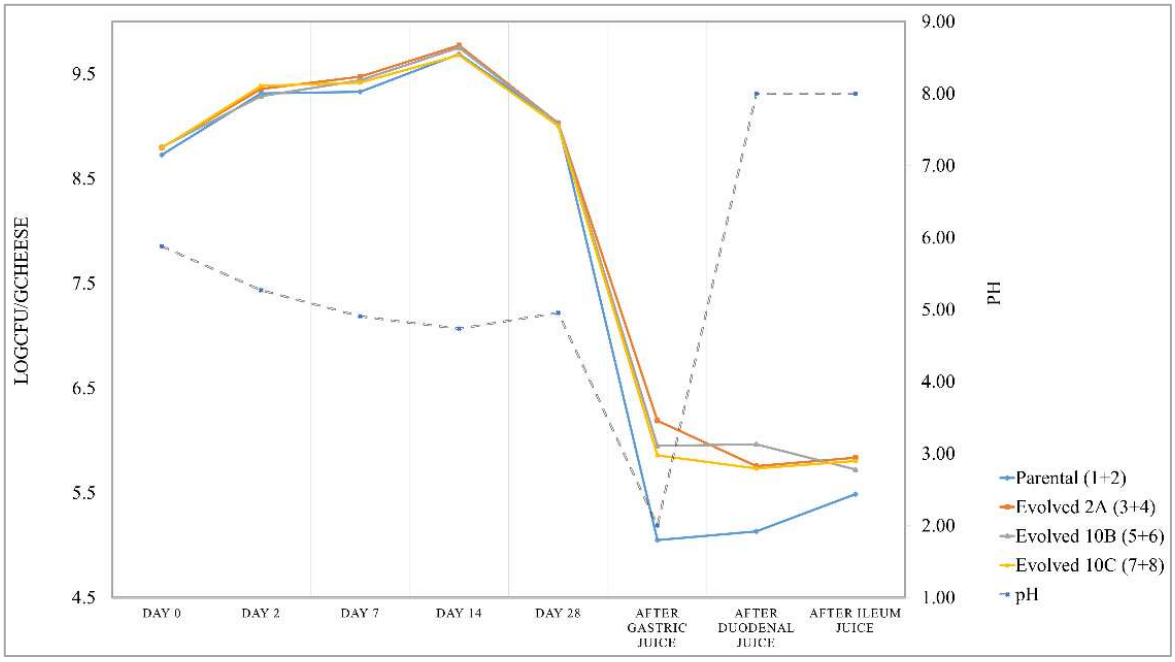

Figure 8. Number of LAB cells (x-axis in CFU/mL) and pH of the microcheeses inoculated with parental and evolved *Lb. plantarum* 1QBMRS77 during the cheese process (with 28 days of ripening) and after simulated gastrointestinal transit conditions.

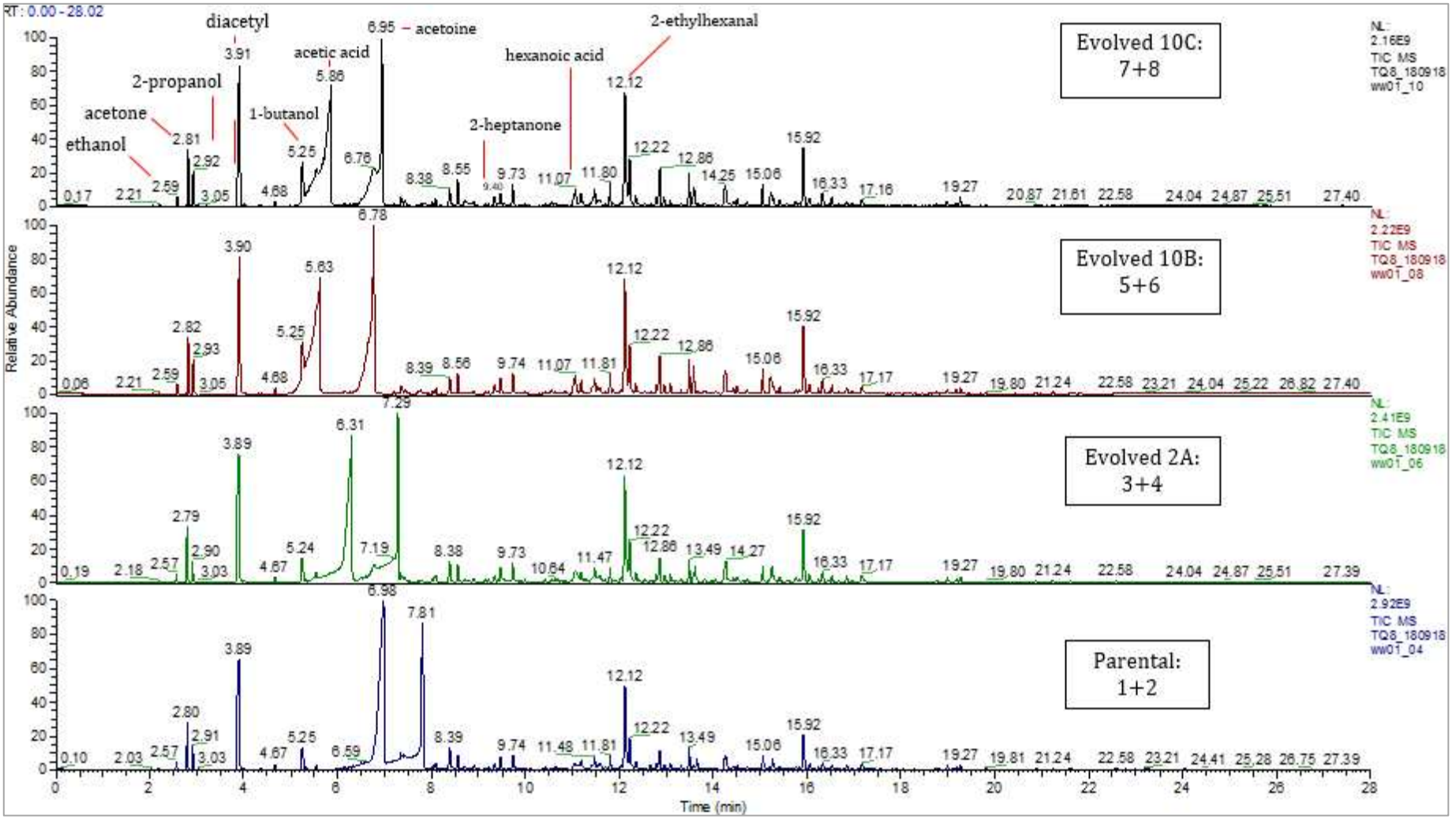

Figure 9. GC/MS chromatogram of head space of microcheeses inoculated with parental (1+2) and evolved strains from tubes 2A (3+4), 10B (5+6) and 10C (7+8).

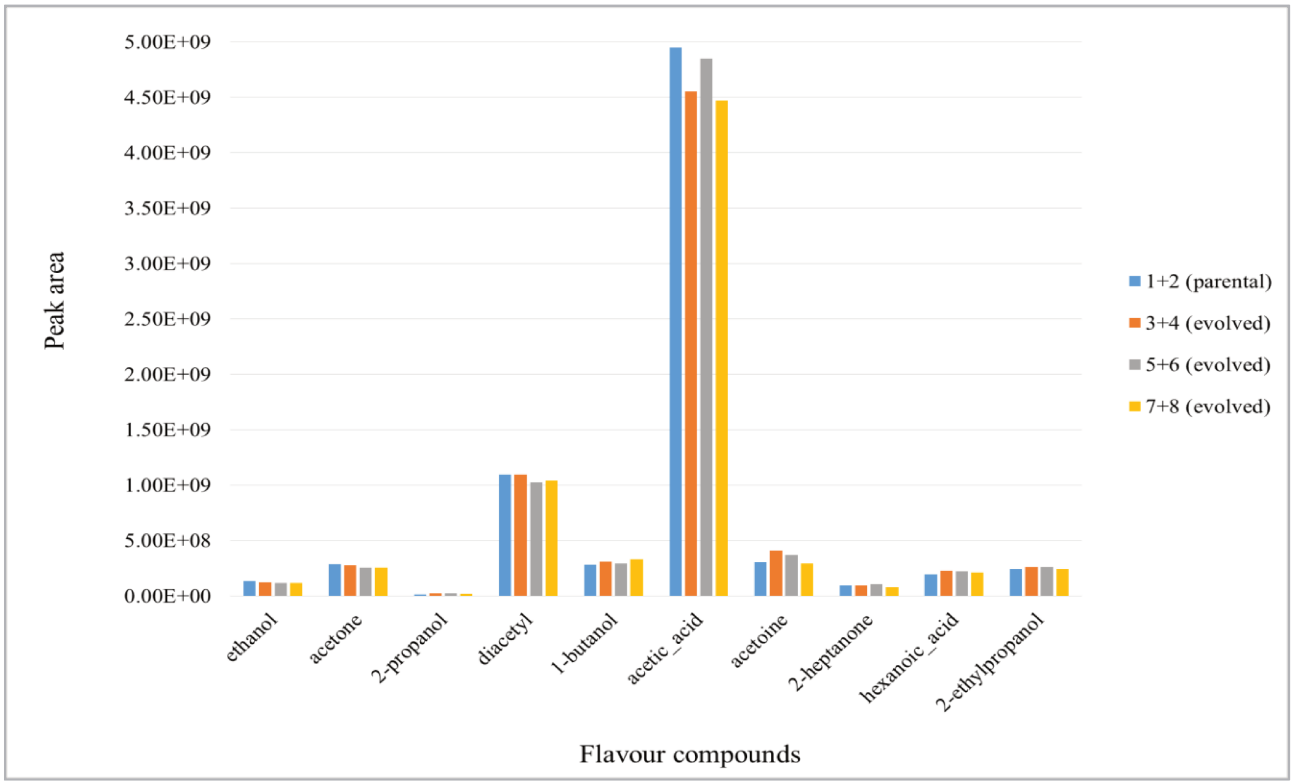

Figure 10. Quantification of volatile flavor compounds as measured in Minas Artisanal microcheese produced with parental (1+2) and evolved strains (3 to 8) of *Lactobacillus plantarum* 1QB77, after 28 days of ripening. The identified compounds are given on the x-axis and peak areas are given on the y-axis.

DISCUSSÃO GERAL

Há diversos tipos de queijos artesanais comercializados no país, cujas característica organolépticas se diferenciam entre sim, devido, principalmente à microbiota particular referente a cada tipo de queijo. Esta microbiota, composta principalmente por bactérias láticas e leveduras, são carreadas à matéria-prima por diferentes fontes (leite cru, utensílios, manipulador, ar) e desempenham papel fundamental na formação de compostos aromáticos, cor, e sabor dos queijos, caracterizando-os quanto à sua localização geográfica, além de ser considerada uma fonte rica para a composição de novos fermentos lácteos (Kamimura et al. 2019; Kamimura et al. 2019).

Neste contexto, o primeiro e o segundo capítulo visaram fazer um *screening* incial de propriedades tecnológicas relevantes do ponto de vista industrial de 1.002 BAL pertencentes aos gêneros *Lactobacillus* sp., *Lactococcus* sp., *Leuconostoc* sp., *Pediococcus* sp. e 576 cepas pertencentes ao gênero *Enterococcus* sp. quanto ao seu potencial para serem aplicadas como *starter* (boas acidificadoras), adjuntas (boas produtoras de enzimas extracelulares, resistentes ao sal) e funcionais (resistência a baixos valores de pH, sais biliares e inibição de patógenos). Os isolados de *Enterococcus* sp. também foram avaliados quanto à sua segurança, sendo submetidos ao crescimento em meios seletivos para averiguar a presença de cepas resistentes à vancomicina e a capacidade de produzir hemolisinas em ágar sangue; etapas justificadas pelo aumento crescente de cepas resistentes a este antibiótico, que está associada com a transferência horizontal de genes de resistência para outras bactérias, inclusive patogênicas, ao longo da cadeia alimentar (Giraffa G. 2002; Lebreton, Willems, and Gilmore 2014; Sanlibaba and Senturk 2018).

Houve uma grande variabilidade intra-espécie nos testes de acidificação, principalmente entre as espécies dos queijos Araxá, Campos das Vertentes e Serro (Sudeste), Caipira (Centro-Oeste) e Coalho (Nordeste), e todos os isolados apresentaram baixas taxas de acidificação, denotando se tratar de espécies consideradas como não iniciadoras (NSLAB). Este grupo de bactérias é capaz de atuar na produção de ácidos graxos livres e peptídeos precursores de aroma, devido a presença de esterases e peptidases, atuando principalmente na etapa de maturação. Geralmente elas apresentam maior resistência às condições desfavoráveis encontradas nesta etapa (baixo pH, baixa atividade de água e altas concentrações de sal), quando comparada com as culturas iniciadoras (Guidone et al. 2014). A análise multivariada de componentes principais possibilitou verificar a formação de grupos distintos, de acordo com as variáveis estudadas, destacando isolados com maior potencial para culturas adjuntas, devido à resistência ao sal e à produção de enzimas proteolíticas e lipolíticas; e grupos com maior potencial para aplicação como biopreservativos, devido à inibiação de *Listeria monocytogenes*.

O gênero *Enterococcus* sp. esteve presente em todos os queijos analisados, com maior prevalênicas em queijos Marajó, coalho e manteiga (75-69.57 %), possivelmente devido à etapa de cozimento (40-60 ºC) aplicada durante o processo de produção. Sabe-se que este gênero é capaz de resistir a temperaturas de 60 ºC por 30 min, o que pode ter selecionado esta espécie em relação às demais. Além disso, por ser uma bactéria ubíqua (dispersa no ambiente) devido à sua fácil capacidade de adaptação, ele está naturalmente presente em alimentos, incluindo queijos (Carvalho 2007; Giraffa and Rossetti 2004; Lebreton, Willems, and Gilmore 2014). Do total de 576 cepas confirmadas como pertencentes ao gênero *Enterococcus*

sp., 63.19 % foram consideradas adequadas para aplicação em fermentos lácteos, abrindo novas possibilidades para este gênero.

A partir do *screening* inicial realizado para 1.002 BAL, foi possível selecionar cepas representativas de cada grupo formado, a fim de dar continuidade à bioprospecção de características relacionadas ao aroma, textura e confirmação da natureza proteica de substâncias inibidoras frente à cepas enterotoxigênicas de *Staphylococcus aureus* e *Listeria monocytogenes*. Observou-se que 131 isolados, pertencentes aos gêneros *Lactobacillus* sp., *Lactococcus* sp. e *Pediococcus* sp, foram classificados como fortes (40,5%) e moderados (19,1%) produtores de diacetil; 28 (12,73%) isolados se destacaram por apresentarem forte formação de exopolissacarídeos a partir de diferentes fontes de açúcar: sacarose (3,2%), frutose (2,3%), lactose (2,3%) e glicose (6%); 94,1 e 95,9% dos isolados apresentaram atividade antagônica frentes os patógenos de *S. aureus* e *L. monocytogenes*, respectivamente, e 27 BAL (12,27 %) apresentaram resultados positivos no teste de produção de bacteriocinas, confirmando a origem proteica da substância antimicrobiana em questão. Nenhuma bactéria apresentou atividade hemolítica e 117 isolados foram classificados como seguros, devido a sua resistência intrínseca a até 4 antibióticos. Os resultados da análise de correspondência mostraram, para as linhagens testadas de BAL, que a primeira e a segunda dimensões englobaram com sucesso 45,27% e 22,17%, respectivamente, explicando 69,37% da variação total no conjunto de dados. Um grande aglomerado compreendendo queijos Araxa, Canastra e Campo das Vertentes e alta atividade de exopolissacarídeos da frutose, glicose e lactose e alta atividade de diacetil foi destacado. As cepas dos queijos Coalho e Caipira se associaram próximo à atividade bacteriocina. Estes dados corroboraram a influência da origem de isolamento na performance dos isolados.

Estas mesmas 220 bactérias também foram investigadas quanto ao seu potencial probiótico, incluindo análises de resistência a condições de estresse encontradas durante a digestão (baixos pH, presença de sais biliares e enzimas digestivas, como pepsinas, pancreatina), às quais 22 isolados foram capazes de resistir; análises de capacidade de colonização por meio de testes de autoagregação, hidrofobicidade e adesão à células Caco-2, cujos resultados foram considerados altos (68,46 – 99,02%), variáveis (4,97-64,25 %) e satisfatórios (> 70 %). Destas 22 cepas, 20 foram classificadas como seguras para aplicação industrial, devido à ausência de genes de virulência e uma cepa de *Lactobacillus plantarum* (1QB77) de queijo Minas artesanal da região do Serro (MG) foi selecionada para aplicação em co-cultura com micro-organismos patogênicos em uma matriz de queijo produzindo em microescala. Foi possível observar uma redução de até 4 unidades logaritmicas (logUFC/g) dos patógenos de *L. monocytogenes* e *S. aureus* do primeiro dia de produção até submissão do microqueijo aos estresses do trânsito gastrointestinal, corroborando o potencial probiótico e biopreservativo da cepa estudada.

Por fim, esta mesma cepa foi submetida a testes de engenharia evolutiva, com o intuito de melhorar o seu potencial de acidificação, a fim de melhorar sua performance frente ao estresse que ocorre na primeira etapa do processo produtivo de queijos (a acidificação). Foi possível observar um aumento de 6 x na taxa de acidificação do leite entre as cepas evoluídas e parentais e uma maior resistência aos estresses do TGI, com taxa de sobrevivência 90 % maior para as cepas evoluídas. Estes dados corroboram a aplicação de técnicas naturais (não-GMO) para o melhroamento de cepas, em adição ao crescente isolamento e bioprospecção de cepas selvagens presentes em produtos tradicionais fermentados.

CONCLUSÕES GERAIS

O presente estudo possibilitou a combinação de métodos de *screening* de alta eficiência com análise multivariada para avaliar o potencial tecnólogico e probiótico de um elevado número de bactérias láticas pertencentes aos gêneros *Lactobacillus* sp., *Lactococcus* sp., *Pediococccus*., *Leuconostoc* sp. e *Enterococcus* sp isoladas a partir de queijos artesanais provenientes das 5 principais regiões do país; As cepas foram classificadas com NSLAB (não-iniciadoras) e foram dividas em grupos, de acordo com seu potencial para aplicação em fermentos lácteos: como culturas adjuntas, probióticas ou biopreservativos;

Houve uma associação de determinados fenótipos com a origem de isolamento, corroborando a influência da região na performance das cepas: Os queijos do Marajó se destacando pela presença de bactérias altamente lipolíticas, e os queijos Minas artesanal pela grande presença de cepas bacteriocigênicas capazes de inibir o crescimento de patógenos veiculados a alimentos lácteos: *Listeria monocytogenes* e *Staphylococcus aureus* produtores de enterotoxinas, podendo contribuir para a fabricação de queijos de maneira mais segura e mantendo a qualidade organolépticas referentes aos queijos artesanais de origem;

A seleção racional de propriedades probióticas, possibilitou a escolha de um isolado de *Lactobacillus plantarum* para aplicação em uma matriz de queijo elaborada em microescala, sendo capaz de resistir e manter elevadas concentrações no início, durante a maturação e após submissão aos estresses do trânsito gastrointestinal; e de reduzir 4 unidades logarítmicas quando inoculados em co-cultura com os patógenos supracitados;

O método de engenharia evolutiva possibilitou o melhoramento de *Lactobacillus plantarum*, aumentado sua taxa de acidificação em leite (em 6x) e

resistência a baixo pH encontrado no suco gástrico (em 1 log), sem interferir nas características organolépticas dos microqueijos.

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ANEXOS

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Comprovante de Cadastro de Acesso

Cadastro nº AB0E472

A atividade de acesso ao Patrimônio Genético, nos termos abaixo resumida, foi cadastrada no SisGen, em atendimento ao previsto na Lei nº 13.123/2015 e seus regulamentos.

Número do cadastro:	**AB0E472**
Usuário:	**LARISSA PEREIRA MARGALHO**
CPF/CNPJ:	**010.773.162-24**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa e Desenvolvimento Tecnológico**

Espécie

4063 Bactérias láticas e leveduras sp

Título da Atividade:	**Bactérias láticas e leveduras em queijos artesanais brasileiros: aplicação da engenharia evolutiva para o melhoramento de culturas visando a aceleração do processo de maturação**

Equipe

LARISSA PEREIRA MARGALHO	**UNICAMP**
ANDERSON DE SOUZA SANT'ANA	**UNICAMP**
HERWIG BACHMANN	**NIZO food research**

Parceiras Nacionais

46.068.425/0001-33 / Universidade Estadual de Camp

Parceiras no Exterior

NIZO food research

Data do Cadastro:	**12/12/2017 07:42:18**
Situação do Cadastro:	**Concluído**

Conselho de Gestão do Patrimônio Genético
Situação cadastral conforme consulta ao SisGen em **10:11** de **14/12/2017**.

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO - SISGEN