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Diversidade e evolução da microbiota lática autóctone em queijo Muçarela de búfala e aplicação tecnológica dos isolados

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

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RESUMO

No Brasil, o queijo Muçarela de búfala tem uma boa aceitação pelos consumidores e mercado em expansão. Entretanto, há escassez de dados científicos em âmbito nacional sobre as bactérias acido láticas (BAL) autóctones envolvidas nos processos tradicionais de fabricação deste produto. Assim, este estudo teve o objetivo de identificar e caracterizar as BAL autóctones isoladas durante as etapas do processo de produção e período de estocagem do queijo Muçarela de búfala, assim como avaliar a dinâmica e a evolução da microbiota lática, suas características tecnológicas e o potencial de aplicação industrial. Para isso, em um primeiro momento, cento e cinquenta e duas culturas láticas isoladas das amostras de leite *in natura*, coalhada, massa filada, queijo Muçarela de búfala e soro de conservação recém processados e durante o período de estocagem foram caracterizadas pela capacidade de crescer em diferentes temperaturas (15, 30 e 45 °C), pH (4,5 e 9,6), concentrações de NaCl (4,0, 6,5 e 10,0%) e pela capacidade de produzir CO_2 a partir do uso da glicose. A biodiversidade e a evolução das BAL foram avaliadas pelo sequenciamento do gene 16S rRNA e pelas técnicas de RAPD-PCR e RFLP-PCR. Quanto à viabilidade nas diferentes condições, a maioria das cepas analisadas cresceu a 30 °C e na presença de 6,5% de NaCl, e em geral, cresceram bem em pH 9,6. As BAL isoladas foram identificadas como: *Enterocccus faecalis*, *Lactococcus garvieae*, *Lactobacillus helveticus*, *Lactococcus lactis*, *Streptococcus thermophilus*, *Leuconostoc citreum*, *Lactobacillus delbrueckii* subsp. *bulgaricus, Enterococcus sp*., *Lactobacillus fermentum*, *Lactobacillus casei* e *Leuconostoc mesenteroides*. A técnica de RAPD-PCR permitiu avaliar a dinâmica das BAL representativas nas diferentes etapas de processamento e no período de estocagem do queijo Muçarela. Sessenta *clusters* foram obtidos pela técnica de RAPD-PCR. Todas as culturas agrupadas pela técnica de RAPD-PCR, por apresentarem mais de 85% de similaridade (114 culturas), foram avaliadas pela técnica de RFLP-PCR. A maioria das BAL foi agrupada com 100% de similaridade pela técnica de RFLP-PCR. Em um segundo momento, as BAL foram caracterizadas pela capacidade de utilizar citrato, pela atividade proteolítica, pela capacidade de reduzir o pH do leite e de produzir ácidos orgânicos, acetoina e diacetil em leite desnatado durante a fermentação, usando *High Performance Liquid Chromatography* (HPLC), seguido da Análise do Componente Principal (ACP). A capacidade de utilizar citrato em meio diferencial foi observada por todas as culturas de *Lc. mesenteroides*, *Lc. citreum*, *L. lactis* e *Lb. fermentum*, bem como por algumas cepas de *Lb. casei*. A maioria das cepas se mostrou capaz de produzir

proteases extracelulares e de reduzir o pH do leite para ≤ 5,0 no final da fermentação, bem como de produzir altas concentrações de compostos orgânicos. As culturas de *Lc. mesenteroides* foram caracterizadas por sua capacidade de produzir ácidos orgânicos (acético, láctico, fórmico e pirúvico) e acetoína em leite desnatado fermentado a 30 °C; as cepas de *Enterococcus* sp. foram caracterizadas pela capacidade de produzir ácidos acético, fórmico e pirúvico e *Lb. casei* pela produção de ácido lático em leite desnatado fermentado a 37 °C; as cepas *Lb. helveticus*, *Lb. bulgaricus* foram caracterizadas pela capacidade de produzir acetoína, enquanto a produção de ácido lático foi relacionada a todas as cepas de *Lb. bulgaricus* e de *St. thermophilus.* As cepas de *Lb. fermentum* foram caracterizadas pela capacidade de produzir ácido acético, fórmico e pirúvico em leite desnatado fermentado a 42 °C. Ainda, para a caracterização do potencial de aplicação, as cepas de *St. thermophilus* autóctones, isoladas das amostras do queijo Muçarela de búfala, foram avaliadas quanto à segurança (genes que codificam fatores de virulência e resistência aos antibióticos) e à atividade acidificante. A cultura considerada segura também apresentou boa atividade acidificante e foi selecionada como cultura *starter* para a fabricação do queijo Muçarela de búfala. A composição química, o perfil de textura e a proteólise dos queijos produzidos usando a cultura autóctone foram avaliados e comparados com as características do queijo comercial (controle). Além disso, as BAL cultiváveis também foram avaliadas pelo método de contagem em placa e o comportamento das culturas de *St. thermophilus* (autóctone e comercial) foi avaliada diretamente da amostra pelo método independente de cultivo (Real Time-qPCR) durante a fabricação do queijo e período de estocagem. A composição química, o perfil textura e a proteólise foram semelhantes para ambos os tratamentos (cultura autóctone e controle). A contagem de BAL foi maior durante o processo de produção do queijo do que no período de estocagem. Adicionalmente, os resultados observados pela técnica de Real time-qPCR indicaram a presença de *St. thermophilus* (autóctone e comercial) durante o processo até ao final do período de estocagem do queijo, indicando a sua robustez para a produção do queijo. Os resultados obtidos neste estudo mostram a biodiversidade e a dinâmica das BAL presentes durante a produção e o período de estocagem do queijo Muçarela de búfala, além de apresentar novas culturas láticas com características tecnológicas interessantes para a aplicação industrial.

Palavras-chave: Produtos lácteos; Biologia molecular; Caracterização tecnológica; Ácidos orgânicos, Viabilidade.

ABSTRACT

In Brazil, buffalo Mozzarella cheese has good acceptance by its consumers and growing market. However, there are few scientific data about autochthonous lactic acid bacteria (LAB) involved in traditional manufacturing processes of this product nationally. Thus, this study aimed to identify the autochthonous LAB isolated during the buffalo Mozzarella cheese manufacture and storage period, as well as to evaluate the evolution and dynamic of lactic microbiota and their technological characteristics and potential of industrial application. First, isolated one hundred fifty-two lactic cultures isolated from raw milk, curd, stretched curd, mozzarella cheese, and solution of maintenance after being produced and during storage were characterized by the ability to grow in different temperatures (15, 30 and 45 °C), pH (4.5 and 9.6) and concentrations of NaCl (4.0, 6.5 and 10.0%), as well as the ability to produce CO_2 from glucose. The biodiversity and evolution of LAB were evaluated by 16S rRNA gene sequencing, RAPD-PCR, and RFLP-PCR techniques. Regarding the growth under different conditions, most of the strains grow well at 30 °C, are feasible in the presence of 6.5% NaCl, and in general, presented best growing in pH 9.6. The isolated LAB were identified as: *Enterocccus faecalis*, *Lactococcus. garvieae*, *Lactobacillus helveticus*, *Lactococcus lactis*, *Streptococcus thermophilus*, *Leuconostoc citreum*, *Lactobacillus delbrueckii* subsp. *bulgaricus, Enterococcus sp*., *Lactobacillus fermentum*, *Lactobacillus casei* and *Leuconostoc mesenteroides*. The RAPD-PCR technique allowed evaluating the dynamics of representative LAB in the different steps of Mozzarella production and storage period. Sixty clusters were obtained by RAPD-PCR. All cultures grouped by RAPD-PCR, which presented more than 85% of similarity (114 cultures), were assessed by RFLP-PCR. Most of the LAB was clustered with 100% similarity by RFLP-PCR technique. In the second part, the LAB were characterized by their ability to utilize citrate, proteolytic activity, ability to reduce milk pH and to produce organic acids, acetoin and diacetyl in skim milk, using High Performance Liquid Chromatography (HPLC), followed by the Principal Component Analysis (PCA). The ability to utilize citrate in differential medium was observed in all strains of *Lc. mesenteroides*, *Lc. citreum*, *L. lactis* and *Lb. fermentum,* and in some strains of *Lb. casei*. Most of the strains showed ability to produce extracellular protease, were able to reduce the pH to ≤ 5.0, and to produce high concentration of organic compounds. *Lc. mesenteroides* strains were characterized by their ability to produce organic acids (acetic, lactic, formic, and pyruvic) and acetoin in skim milk at 30 °C; *Enterococcus* sp. strains by their ability to

produce acetic, formic, and pyruvic acids and *Lb. casei* strains by the production of lactic acid in skim milk at 37 °C; *Lb. helveticus* and some *Lb. bulgaricus* strains were characterized by their ability to produce acetoin, while the production of lactic acid was related to all *Lb. bulgaricus* and *St. thermophilus* strains, and the production of acetic, formic, and pyruvic acids was a feature of *Lb. fermentum* in skim milk fermented at 42 °C. Additionally, for the characterization of potential application, the *St. thermophilus* strains isolated from buffalo Mozzarella cheese samples were evaluated for safety (genes encoding virulence factors and antibiotic resistance) and acidifying activity. The culture considered safe also showed good acidifying activity, and therefore it was selected as a starter culture for the buffalo Mozzarella cheese production. The chemical composition, texture profile, and proteolysis of cheese produced using the autochthonous culture were evaluated and compared with commercial cheese properties (control). Furthermore, the cultivable LAB were also assessed by plate counts and the behavior of *St. thermophiles* strains (autochthonous and commercial) were directly assessed by culture-independent method (Real Time-qPCR) during cheese manufacture and storage period. The properties of the chemical composition, texture and proteolysis were similar for both cheese. Counts of LAB were higher during cheese manufacture than during storage period. Furthermore, the results of Real time-qPCR method indicated the presence of *St. thermophilus* (autochthonous and commercial) during processing until the end of cheese storage period, demonstrating their robustness for cheese production. The results obtained in this study show the biodiversity and the dynamics of LAB present during the production of buffalo Mozzarella cheese and storage period, besides presenting novel lactic cultures with interesting technological characteristics for industrial applications.

Keywords: Dairy products; Molecular biology; Technological characterization; Organic acids, Viability.

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INTRODUÇÃO

A produção de leite de búfalas no Brasil tem aumentado consideravelmente nos últimos 10 anos, somando hoje um total de 1,5 milhões de bubalinos em território nacional. Anualmente, os laticínios brasileiros produzem em média 18,5 mil toneladas de derivados do leite de búfala. Cerca de 70% desses derivados é representado pelo queijo Muçarela de búfala, o qual apresenta grande potencial de ampliação de mercado e de otimização do processamento, visando a melhoria na qualidade e a redução de custos.

O queijo Muçarela imerso em soro é o principal derivado de leite de búfala consumido pelos brasileiros, principalmente em pizzas ou como aperitivo. Este queijo é caracterizado por uma massa filada, com alta umidade e alto teor lipídico (chegando até 8,59% de gordura total), apresenta corpo macio e coloração branca porcelanada. É elaborado seguindo a tecnologia de produção italiana, que usa culturas láticas no processo de fermentação. O valor nutricional, aliado ao sabor do queijo Muçarela de búfala, faz com que este alimento esteja presente na dieta de consumidores de ampla faixa etária.

As características sensoriais dos queijos são decorrentes principalmente da ação do complexo enzimático das culturas acidoláticas sobre os carboidratos, gorduras e proteínas constituintes da matriz deste produto. As bactérias acidoláticas mesófilas, tais como *Lactococcus lactis* e *L. lactis* subsp. *cremoris*, e termófilas, tais como S*treptococcus thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus* e *Lactobacillus helveticus,* são dominantes nas culturas utilizadas na elaboração do queijo Muçarela, participando da coagulação, acidificação e proteólise da caseína, conferindo-lhe textura, aroma e sabor.

Diversos estudos têm sido dedicados em isolar e caracterizar a microbiota lática do seu habitat natural, com o objetivo de investigar o potencial de aplicação desses micro-organismos. No entanto, embora a microbiota lática desempenhe importante contribuição à tecnologia de fabricação do queijo Muçarela de búfala, em nível nacional, há escassez de dados científicos sobre os aspectos morfo-fisiológicos, tecnológicos e principalmente, sobre as características genotípicas das espécies de bactérias acidoláticas (BAL) envolvidas no processo, o que justifica a necessidade deste estudo. Considerando a taxa de crescimento da produção de leite de búfala e o interesse dos produtores na melhoria da qualidade dos produtos, os resultados obtidos poderão colaborar com o desenvolvimento da indústria brasileira de queijos, na busca de soluções inovadoras para a melhoria da qualidade tecnológica dos queijos de massa filada.

Além disso, o isolamento e a caracterização das bactérias acidoláticas presentes no queijo Muçarela de búfala poderá resultar em novas alternativas de culturas *starters* e/ou culturas adjuntas a serem utilizadas no processo tecnológico de diferentes produtos lácteos.

APRESENTAÇÃO DO TRABALHO

Este trabalho foi organizado em cinco capítulos para a melhor distribuição e entendimento dos assuntos abordados. O Capítulo I consiste em uma Revisão Bibliográfica do tema abordado na tese. Os capítulos II, III e IV foram redigidos na forma de artigos científicos. Estes artigos serão submetidos à publicação em periódicos internacionais classificados pelo Qualis. O capítulo V apresenta a considerações finais desse trabalho.

OBJETIVOS

Objetivos gerais:

- Este estudo teve como objetivo identificar e avaliar a dinâmica e a evolução da microbiota lática autóctone isolada durante as etapas do processo de produção e o período de estocagem do queijo Muçarela de búfala, bem como, avaliar as suas características tecnológicas e o potencial de aplicação industrial.

Os objetivos específicos dos capítulos foram:

CAPÍTULO I

- Revisar os aspectos gerais sobre a produção do queijo Muçarela de búfala, bem como, as características gerais, o isolamento e a identificação das bactérias acidoláticas comumente presentes neste queijo.

CAPÍTULO II

- Caracterizar as culturas isoladas quanto à viabilidade em diferentes temperaturas (15 ºC, 30 ºC e 45 ºC), em diferentes teores de NaCl (4,0%, 6,5% e 10,0%), em pH 4,5 e 9,6 e pela capacidade de produzir CO_2 a partir do uso da glicose; identificar as culturas láticas isoladas do queijo Muçarela de búfala pelo sequenciamento do gene 16S rRNA; avaliar a evolução das culturas láticas presentes em amostras coletadas em diferentes etapas do processamento e período de estocagem do queijo Muçarela de búfala.

CAPÍTULO III

- Caracterizar as bactérias acidoláticas isoladas pela capacidade de utilizarem o citrato, pela atividade proteolítica e pela capacidade de reduzirem o pH do leite e de produzirem ácidos orgânicos, acetoína e diacetil.

CAPÍTULO IV

- Avaliar a segurança (presença de genes que conferem virulência, resistência a antibióticos e produção de aminas biogênicas) e a atividade acidificante das culturas de *Streptococcus thermophilus* isoladas; produzir queijos Muçarela de búfala em escala industrial utilizando a cultura caracterizada como segura e com melhor atividade acidificante; avaliar as características físico-químicas e de textura dos queijos, assim como avaliar a viabilidade das bactérias acidoláticas.

CAPÍTULO V

- Considerações finais

Capítulo I

Revisão bibliográfica

CAPÍTULO I

REVISÃO BIBLIOGRÁFICA

1. Queijo Muçarela

1.1. Aspectos gerais sobre o leite de búfala

Embora os países asiáticos e a Itália sejam os principais produtores do leite bubalino, no Brasil, a sua produção vem aumentando cerca 45% anualmente (ABCB, 2009, SBA, 2012). Segundo o Ministério da Agricultura, Pecuária e Abastecimento (2012), o rebanho brasileiro de bubalinos está estimado em torno de 1,5 milhões, sendo a região Norte a maior produtora do país, com 720 mil animais. Em seguida aparece o Nordeste e o Sudeste, com 135 e 104 mil cabeças, respectivamente.

No Brasil, o consumo do leite de búfala *in natura* é restrito, devido principalmente ao alto teor de gordura, que dificulta a assimilação do leite bubalino pelo organismo, além disso, esta matéria-prima também é considerada ácida (19 °D) (FERREIRA et al., 1995). Por este motivo, a Secretaria de Inspeção de Produtos de Origem Animal (SIPA), através da Portaria de n° 236, permite a adição máxima de 30% do leite bovino ao bubalino, com intuito de sua normalização (FURTADO, 1990).

Comparado ao leite bovino, o leite bubalino apresenta uma percentagem mais elevada em todos os componentes (ABCB, 2009). Apesar do seu alto percentual de gordura, o índice de colesterol do leite e da Muçarela de búfala é menor em relação ao leite e queijo bovino. A composição do leite de búfala possui características próprias, que alteram conforme o período de lactação, raça, alimentação e localização geográfica (BRESCIA et al., 2005; ISLAM et al., 2014).

Em geral, o leite bubalino apresenta 1,025 a 1,047 g/mL de densidade, 6,41 a 6,47 de pH, 14 a 20 °D de acidez (devido ao elevado teor de proteínas, em especial a caseína), 15,64 a 17,95% de sólidos totais e 0,79 a 0,83% de sais minerais (sendo até 25% deste, composto por cálcio) (ZICARELLI, 2004). A composição de 3,91 a 4,55% de proteína e de 6,87 a 8,59% de gordura, em relação ao leite de outras espécies (TONHATI; MUÑOZ; OLIVEIRA, 2000), permite que seu uso seja uma alternativa economicamente mais favorável para a produção de

queijos, entre outras variedades de produtos lácteos. Microbiologicamente, a qualidade do leite de búfala está intimamente relacionada aos hábitos do animal, clima e ao manejo de ordenha (TEIXEIRA; BASTIANETO; OLIVEIRA, 2005).

Além disso, o leite de búfala apresenta inúmeras propriedades biológicas, destacando a concentração (1,77%) de acido linoleico conjugado (CLA) (um intermediário do processo de biohidrogenação do acido linoleico por bactérias ruminantes), sendo o isômero cis-9 trans-11 reconhecido por sua capacidade anticarcinogênica e prevenção de doenças cardiovasculares, o que permite inclui-lo no grupo denominado alimento funcional, de grande e crescente demanda pelos consumidores. Além disso, o leite de búfala, em comparação ao leite de vaca, apresenta também maiores teores de vitamina A e de cálcio (MONTREZOR, 2006).

As proteínas do leite de búfala (caseínas, albuminas e globulinas) são similares àquelas do leite de vaca, porém não são idênticas e não são encontradas nas mesmas proporções. Estudos recentes têm sido realizados a fim de quantificar as frações da caseína e das proteínas do soro além de compará-las com essas características no leite bovino (BUFFONI et al., 2011; BONFATTI et al., 2013). As proteínas do leite bubalino e bovino apresentam menores diferenças nas frações α_{s1}- e β-caseína se comparado às frações α-caseína e α_{s2}-caseína (Quadro 1). Pesquisas na Rússia demonstraram que a caseína do leite de búfalas apresenta 22% mais aminoácidos essenciais do que a caseína do leite bovino (TEIXEIRA; BASTIANETO; OLIVEIRA, 2005). A composição proteica foi representada por 32,2% de α_{s1}-, 15,8% de α_{s2}-, 36,5% de β- e 15,5% de κ-caseína do teor total de caseína, enquanto a β-lactoglobulina foi 1,3 vezes mais elevada que a α-lactalbumina na avaliação realizada por Bonfatti e colaboradores (2013).

A maior quantidade de κ-caseina presente no leite bubalino acelera a coagulação enzimática, o que requer menor quantidade de renina para a produção dos queijos (ZICARELLI, 2004; ISLAM et al., 2014). Dessa forma, o ponto ótimo para a filagem de queijo Muçarela elaborado com o leite de búfala pode ser obtido com a coalhada em pH 4,9, enquanto com o leite bovino, em pH 5,0-5,2 (ADDEO; EMALDI; MASI, 1996).

Quadro 1 Proporção das frações de caseína/caseína total entre leite bovino e bubalino (ZICARELLI, 2004).

Frações da caseína	Búfala (%)	Vaca (%)	Búfala/Vaca (%)
α_{s1}	30,2	38,4	78,6
α_{s2}	17,6	10,5	167,6
β	33,9	36,5	92,9
K	15,4	12,5	123,9
Total	97,1	97,9	
$\alpha_{s1} + \alpha_{s2}$	47,8	48,9	97,7

A composição de aminoácidos da κ-caseína do leite de búfalas e vacas difere na quantidade (mol/mol de proteína) de N-acetilgalactosamina (0-4,3 e 0-6,7) e de ácido siálico (5,5-8,5 e 3,5-4,3), respectivamente (ZICARELLI, 2004). Em leite bovino, a α_{s1}-caseína tem um grupo fosfoseril na posição 115, que é envolvido por aminoácidos hidrofóbicos. Provavelmente, a interação desses grupos hidrofóbicos é responsável pelas características da fração α_{s1}-caseína nas micelas. A ausência desse grupo na fração α_{s1}-caseína no leite bubalino reforça o caráter não-polar da proteína (FERRANTI et al., 1998). Além disso, a ausência do grupo fosfato, o aumento na densidade e a sensibilidade das micelas de caseína pela quimosina no leite bubalino podem explicar, em parte, a redução no tempo de coagulação e o maior rendimento do queijo elaborado com leite de búfala (ADDEO; MERCIER; RIBADEAU-DUMAS, 1980). A β-caseína do leite bubalino apresenta duas variantes (β_A e β_B) (FERRANTI et al., 1998), que se assemelham à β_{A2}-caseína do leite bovino, diferindo por quatro e cinco aminoácidos, respectivamente. As frações β e α_{s1} da caseína compõem 70% da rede micelar proteica. A hidrólise da β-caseína do leite bubalino pela plasmina, produz as frações γ_2 e γ_3 (ZICARELLI, 2004), sendo que a fração γ_2 atua como marcador, permitindo detectar a presença (<1%) de leite bovino em queijo Muçarela de búfala.

1.2. O queijo Muçarela de búfala

O queijo Muçarela tradicional é elaborado com leite de búfala, tem origem italiana e é habitualmente produzido na região de Campânia, no sul do país e recebeu certificação européia designada PDO (*Product of Designated Origin*, regulamento nº 1107) em junho de 1996, a fim de proteger sua integridade (DE CANDIA et al., 2007). Nos últimos anos, este derivado lácteo tem sido também exportado e extensivamente produzido por outros países

(CECCHINATO et al., 2012; ERCOLINI et al., 2012), inclusive no Brasil. Segundo a Associação Brasileira de Criadores de Búfalos (ABCB, 2009), 70% de todo o leite de búfala produzido no país é direcionado a produção do deste queijo.

Entende-se por Muçarela o queijo obtido pela filagem de uma massa acidificada (produto intermediário obtido por coagulação de leite por meio de coalho e/ou outras enzimas coagulantes apropriadas), complementada pela ação de bactérias lácticas específicas. É um queijo de média, alta ou muita alta umidade e extragordo, gordo ou semigordo e com denominações de "queijo mussarela", "queijo muzzarella" ou "queijo mozzarela" (BRASIL, 1997).

No Brasil, o queijo Muçarela é considerado o queijo mais popular e tradicionalmente apresenta consistência suave, textura fibrosa e elástica, de coloração branca e uniforme, com sabor lático e odor suave, não apresentando olhaduras, devendo ser conservado sob refrigeração em temperaturas positivas de até 10 °C. Apresenta-se de várias formas, como: barra, manta, palito, nozinho, trança e a tradicional bola, comumente comercializada imersa em soro, sendo própria para consumo em, no máximo, 30 dias após a fabricação (ERCOLINI et al., 2004; ABCB, 2009).

Por originar-se de matéria-prima rica em nutrientes, o rendimento da muçarela elaborada com leite de búfala é superior (podendo superar em aproximadamente 40%) à obtida a partir de leite bovino (BASTIANETTO; ESCRIVÃO; OLIVEIRA, 2005). O sabor levemente adocicado e acidificado, aliado ao valor nutritivo, faz do queijo Muçarela de búfala um produto bem aceito pelo consumidor (MACEDO et al., 2001).

1.3. Tecnologia de produção e qualidade

O queijo Mussarela de búfala italiano é produzido tradicionalmente utilizando leite *in natura* adicionado de soro fermento (ERCOLINI et al., 2012) ou utilizando leite pasteurizado com a adição de culturas *starters* comerciais de bactérias acidoláticas (BAL) (COPPOLA et al., 2001). O soro fermento é uma cultura microbiana natural resultante da dessora advinda da coagulação do leite, o qual é armazenado e utilizado como "cultura *starter*" em processos subsequentes (ERCOLINI et al., 2004). Em queijos elaborados com o uso de soro fermento, os micro-organismos presentes durante a coagulação da massa são considerados os responsáveis pela fermentação e acidificação do leite, com a consequente liberação de

compostos importantes que influenciam a textura e o sabor do produto final (MAURIELLO et al., 2001, MAURIELLO et al., 2003; SANTARELLI et al., 2013).

No Brasil, o processo comumente utilizado na elaboração do queijo Muçarela usando leite de búfala é semelhante ao usado na fabricação com leite bovino. As etapas de processamento envolvem, geralmente, a pasteurização do leite, adição de coalho e de culturas láticas iniciadoras (*starters*) termofílicas, a coagulação e fermentação da massa, que propicia a redução do pH e a precipitação da coalhada (VERRUMA-BERNARDI et al, 2000). Em seguida, ocorre a etapa de corte do coágulo e dessora, filagem (fusão da massa em água quente) até a obtenção de uma massa macia e homogênea, corte da massa (opcional), moldagem, resfriamento da massa e envase. Em alguns casos isolados, utiliza-se leite *in natura* e a fermentação ocorre pela adição de soro fermento ou de forma espontânea e incontrolada, pela ação de BAL autóctones presentes no leite, utensílios e no ambiente de produção (SILVA et al., 2015).

A etapa de pasteurização deve ser feita para garantir que o leite fique isento de micro-organismos prejudiciais à saúde e ocorra a redução de micro-organismos deteriorantes. Após a pasteurização é necessário o manuseio atendendo muitos cuidados higiênicos para evitar a recontaminação do leite. A pasteurização pode ser feita por dois processos: lento e rápido. A pasteurização lenta consiste no aquecimento do leite até 65 °C, mantendo-o nessa temperatura por 30 minutos. Para grandes volumes de leite, a pasteurização ocorre em pasteurizadores de placas, no qual o leite é aquecido a 72-75 °C, durante 15 segundos (FURTADO, 1997).

Quando o leite passa pela a etapa de pasteurização, ocorre também a eliminação de micro-organismos responsáveis pela fermentação da massa do queijo, sendo assim necessária a adição das culturas *starters*. Atualmente, uma dificuldade é que a indústria brasileira é dependente de culturas *starters* importadas, comercializadas por representantes de empresas estrangeiras, que nem sempre atendem os requisitos desejados.

Após a etapa de pasteurização, o leite deve ser imediatamente resfriado a 34 °C para ocorrer a etapa da coagulação, que pode ocorrer por acidificação (produção de ácidos orgânicos pelas culturas láticas ou por acidificação direta por meio da adição de ácidos), ou por coagulação enzimática, empregando o coalho. Na acidificação ocorre redução do pH, desmineralização da proteína e formação da coalhada. A coagulação enzimática ocorre à temperatura de 35-37 °C em duas etapas: a hidrólise da κ-caseína e liberação do glicomacropeptídeo, e a reação da κ-caseína hidrolisada (para- κ-caseína) com cálcio, formando o para-caseinato de cálcio (coalhada). A coagulação ácida é mais demorada e

depende da ação das culturas láticas empregadas, enquanto que a coagulação enzimática da massa ocorre após 40-50 minutos da adição do coalho (PERRY, 2004). Tradicionalmente os coalhos são proteases de origem animal, principalmente de bezerros e suínos, mas também podem ser proteases obtidas por fermentação fúngica ou extraídas de plantas.

Após a coagulação da massa é realizado o corte do coágulo, usando-se uma lira vertical e/ou uma horizontal. É importante que os cubos tenham tamanhos bem aproximados, para que a retirada do soro do coágulo seja homogênea, caso contrário há risco de perda de qualidade do produto. A fragmentação irregular da massa reduz o rendimento e pode ocasionar defeitos nos queijos, pois a intensidade do corte está relacionada com a saída do soro (sinérese), resultando em queijos com umidade irregular (FURTADO, 1997).

Após o corte do coágulo, a massa deve manter-se em repouso (com alguns intervalos rápidos de agitação lenta) para que ocorra a fermentação pelas culturas láticas até pH entre 4,9-5,2 (pH ótimo para a filagem da massa). A etapa de fermentação comumente ocorre a 42 °C utilizando culturas láticas termofílicas. Nesse caso, as bactérias acidoláticas são capazes de se desenvolverem em meio ácido, permitindo uma fermentação bem sucedida (até pH final) entre 4-5 horas (culturas termofílicas). No caso do uso de culturas mesofílicas ou do uso de soro fermento, a fermentação pode ocorrer em até 24 horas, enquanto a fermentação espontânea, a partir do leite *in natura*, pode ocorrer em mais de 24 horas (FURTADO, 1997).

Embora do ponto de vista econômico não seja interessante o uso de leite *in natura* devido, principalmente, a veiculação de contaminantes, certos queijos elaborados a partir de leite *in natura* apresentam características sensoriais particulares comparados aos que são produzidos com leite pasteurizado (FRANCIOSI et al., 2009; FERNANDEZ et al., 2010). Estas características estão diretamente relacionadas com as propriedades fisiológicas e bioquímicas das BAL autóctones presentes no leite, que são responsáveis pela fermentação e qualidade do produto (FRANCIOSI et al., 2009). As características sensoriais são manifestadas em decorrência da fermentação de lactose e da hidrólise das proteínas. Assim, além do sistema proteolítico ser essencial para o desenvolvimento das BAL no leite, a ação desses micro-organismos sobre as proteínas do leite pode resultar em pequenos peptídeos e aminoácidos livres, liberados pela hidrólise, precursores do sabor e aroma peculiares (McSWEENEY; SOUSA, 2000).

Durante o processo de fermentação da massa no soro, a proliferação das BAL presentes resulta em rápida acidificação do soro com ação antimicrobiana e em proeminente proteólise secundária, produzida por proteinases e endo e exo-peptidases celulares (DE

SIMONE et al., 2009). Assim, ocorre em uma série de eventos devida à ação combinada de proteases do leite, enzimas responsáveis pela coagulação e a ação das culturas láticas (CORTEZ et al., 2008).

Posteriormente à fermentação da massa, ocorre a dessora e a filagem da massa. A etapa de filagem consiste em fusão da massa realizada em água aquecida a 80-85 °C e esticamento/amassamento até que se torne elástica, permitindo a formação de fios compridos de massa macia e homogênea. Em pequenas produções, a filagem pode ser feita manualmente; em produção industrial, geralmante utilizam-se equipamentos que executam a trituração e filagem mecânica da massa (FURTADO, 1997).

Terminada a filagem, a massa é moldada no formato desejado e, em seguida, submersa em água gelada para ser resfriada. A salga (opcional) da massa pode ser feita em solução a 20% de NaCl (FURTADO, 1991; TEIXEIRA; BASTIANETTO; OLIVEIRA, 2005). A tradicional Muçarela em formato de bola é usualmente moldada e estocada imersa em "solução de estocagem" composta por solução NaCl e/ou $CaCl_2$, denominada salmoura ou soro (LUCERA et al., 2014).

A qualidade do queijo Muçarela de búfala pode ser avaliada pela sua composição microbiológica e físico-química. Em geral, os parâmetros químicos e microbiológicos são estabelecidos pela legislação de cada país. Por exemplo, no Brasil a legislação (BRASIL, 1997) permite no máximo 10^4 UFC/g de coliformes, 10^3 UFC/g coliformes termotolerantes e 10^3 UFC/g de estafilococos coagulase positiva. Não é aceitável a presença de *Salmonela* spp. e *Listeria monocytogenes*. A umidade não deve ultrapassar 60 g/100g e a gordura no extrato seco deve ser no máximo de 35 g/l00g. Vários métodos, incluindo avaliações sensoriais e instrumentais, podem ser utilizados para avaliar as propriedades físicas do queijo Muçarela de búfala.

2. Bactérias acidoláticas (BAL)

As BAL foram isoladas primeiramente de leite *in natura* (CARR; CHILL; MAIDA, 2002; CHERIGUENE et al., 2007), mas têm sido encontradas em alimentos de diferentes fontes e em produtos fermentados, tais como produtos cárneos, produtos lácteos, vegetais, bebidas, entre outros (LIU, 2003; CHERIGUENE et al., 2007; BEN BELGACEM et al., 2009; LEITE et al., 2013; MANGIA et al., 2014), bem como em silagens e no trato intestinal de animais e humanos (BRUNO; CARVALHO, 2009).

Com exceção dos *Enterococcus* sp., as BAL foram reconhecidas como seguras (GRAS - *generally recognized as safe*) pelo FDA (U.S. *Food and Drug Administration*, http://www.fda.gov/), por sua longa tradição de uso nos processos fermentativos de diversos alimentos sem trazer danos à saúde (GASPAR et al., 2013).

O termo BAL refere-se, principalmente, ao metabolismo basal dessas bactérias, como a fermentação de hexoses, produzindo, principalmente, o ácido lático (DE DEA LINDNER, 2008). Nos produtos lácteos, o metabolismo e a interação entre as diferentes linhagens são responsáveis pela produção de ácidos e a coagulação do leite, assim como, para o desenvolvimento de outros componentes, que em conjunto, fornecem propriedades sensoriais específicas aos produtos fermentados.

O grupo das BAL é bastante heterogêneo quando se refere à capacidade de utilizarem o oxigênio, sendo estas classificadas como anaeróbias facultativas, anaeróbias e microaerófilas. De acordo com a temperatura ótima de crescimento, as BAL são divididas em mesófilas com crescimento entre 20°C–30°C e termófilas com crescimento entre 37°C–45°C; contudo, compartilham de características fenotípicas particulares (GASPAR et al., 2013). BAL são bactérias Gram-positivas, não esporogênicas, catalase e oxidase negativa (BOTINA; TSYGANKOV; SUKHODOLETS, 2006; DE DEA LINDNER, 2008) e não possuem citocromos. São consideradas extremamente fastidiosas pela exigência de nutrientes específicos, tais como aminoácidos e vitaminas (SALMINEN; VON WRIGHT; OUWEHAND, 2004), apresentam morfotipos bacilares ou em cocos, e caracterizam-se por apresentar tolerância ao ácido.

As bactérias Gram-positivas são divididas em dois grandes grupos filogenéticos *Clostridium* e *Actinomycetes*, com base em comparações do conteúdo de guanina + citosina (G + C) da sequencia conservada do DNA ribossomal. O grupo *Clostridium* abrange as bactérias cuja composição do DNA contém baixo conteúdo do par G + C. O grupo *Actinomycetes* compreende as bactérias com conteúdo G + C superior a 50%. As bactérias do gênero *Bifidobacterium* exibem um conteúdo relativamente elevado de guanina + citosina (G+C) e, portanto, pertencem ao grupo *Actinomycetes.* Por outro lado, os gêneros *Lactobacillus, Lactococcus, Enterecoccus, Streptococcus, Pediococcus* e *Leuconostoc* contém baixo conteúdo de G + C e estão incluídos no grupo *Clostridium*. Apesar disto, as bifidobactérias compartilham características fisiológicas, bioquímicas e também alguns nichos ecológicos, como o trato gastrointestinal, com as BAL típicas (VASILJEVIC; SHAH, 2008, FRANÇOISE et al., 2010).

De acordo com a taxonomia atual, BAL pertencem ao filo Firmicutes, classe *Bacilli* e ordem *Lactobacillales*, as quais pertencem às famílias das *Aerococcaceae, Carnobacteriaceae, Enterococcaceae, Leuconostocaceae, Lactobacillaceae e Streptococcaceae* (GASPAR et al., 2013).

São considerados do grupo das LAB os micro-organismos dos gêneros *Aerococcus, Alloiococcus, Carnobacterium, Dolosigranulum, Enterococcus, Globicatella, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Melissococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weisella* (CROWLEY; MAHONY; VAN SINDEREN, 2013).

Quanto ao metabolismo, as BAL podem ser homofermentativas e heterofermentativas. As BAL homofermentativas utilizam a via Embden-Meyerhof-Parnas (glicolítica) para metabolizar a glicose (principal fonte de carbono), principalmente, em ácido lático como produto final. Essa fermentação é denominada homolática e resulta em ganho líquido de duas moléculas de ATP e duas moléculas de ácido lático por molécula de glicose fermentada (SALMINEN; VON WRIGHT; OUWEHAND, 2004). As BAL heterofermentativas utilizam a via da fosfocetolase para produzir, além do ácido lático, diversos produtos, incluindo CO_2, ácido acético e etanol, a partir da fermentação da glicose (CARR; CHILL; MAIDA, 2002; GASPAR et al., 2013). Essa fermentação é denominada heterolática e resulta em ganho líquido de uma molécula de ATP e uma molécula de cada um dos produtos finais por molécula de glicose fermentada (SALMINEN; VON WRIGHT; OUWEHAND, 2004). As vias metabólicas dos monossacarídeos estão representadas nas Figuras 1a e 1b.

Os Lactobacilos, o maior grupo de BAL, são classificados de acordo com a capacidade de utilizar fontes de carbono em (SALMINEN; VON WRIGHT; OUWEHAND, 2004):

- Grupo I - lactobacilos homofermentativos obrigatórios - fermentam as hexoses quase que exclusivamente para a produção de ácido lático (85%) pela via de Embden-Meyerhof-Parnas;
- Grupo II - lactobacilos heterofermentativos facultativos - fermentam hexoses também via de Embden-Meyerhof-Parnas para a produção de ácido lático, mas são capazes de degradar pentoses e gluconato pela via da fosfocetolase, resultando na produção de ácido acético, etanol e ácido fórmico, quando há limitações da glicose;
- Grupo III - lactobacilos heterofermentativos obrigatórios - metabolizam as pentoses e hexoses pela via da fosfogluconato e produz ácido lático, etanol (ou ácido acético) e CO_2.

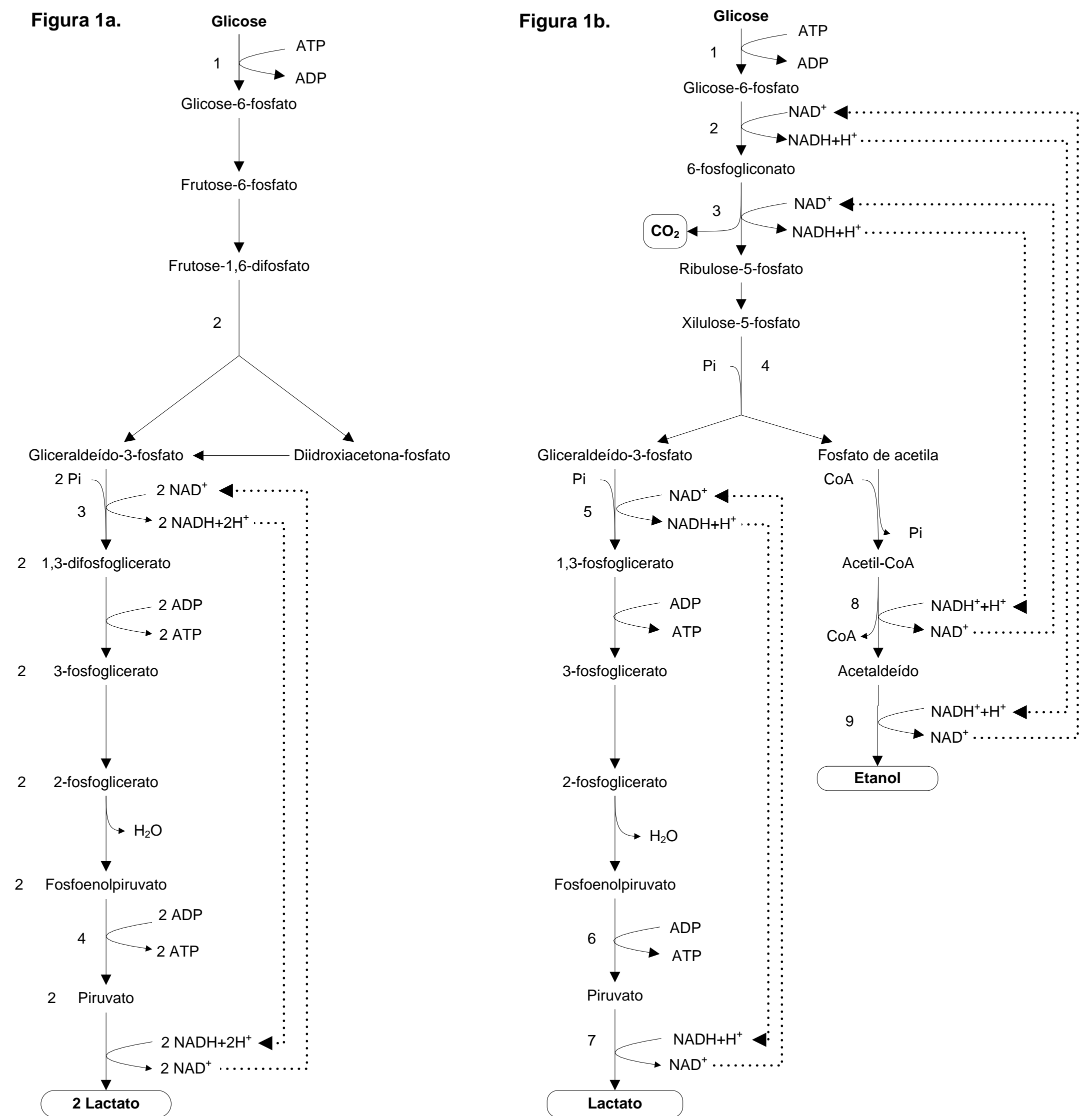

Figura 1. Vias de fermentação das hexoses (Adaptado de SALMINEN; VON WRIGHT; OUWEHAND, 2004). **(a)** – Fermentação homolática (via glicolítica de Emden-Meyerhof-Parnas). 1: glicoquinase; 2: frutose 1,6-difosfato aldolase; 3: gliceraldeído-3-fosfato desidrogenase; 4: piruvato quinase; 5: lactato desidrogenase. **(b)** – Fermentação heterolática (via da 6-fosfogluconato/fosfoacetolase). 1: glicoquinase; 2: glicose-6-fosfato desidrogenase; 3: 6-fosfogliconato desidrogenase; 4: fosfoacetolase; 5: gliceraldeído-3-fosfato desidrogenase; 6: piruvato quinase; 7: lactato desidrogenase; 8: acetaldeído desidrogenase; 9: álcool desidrogenase.

2.1. Segurança das BAL

Embora sejam consideradas seguras e benéficas à saúde do homem, a investigação da segurança das BAL tem sido intensificada nos últimos anos, principalmente por apresentarem fatores de virulência e algumas vezes estarem relacionadas ao desenvolvimento de doenças.

Em geral, as pesquisas de fatores de virulência incluem a presença de genes de virulência, aminas biogênicas e resistência a antibióticos (JERONYMO-CENEVIVA et al., 2014). Os estudos genéticos de virulência incluem principalmente os genes relacionados à proteína de superfície, lise e adesão celular (TODOROV et al., 2011).

Os genes comumente presentes em BAL relacionados com a formação de aminas biogênicas são: histina descarboxilase, tirosina descarboxilase e ornitina descarboxilase (VANKERCKHOVEN et al., 2004; MARTIN-PLATERO et al., 2009). A descarboxilação dos aminoácidos presentes na matriz dos queijos resulta na formação de aminas biogênicas correspondentes: histamina, tiramina e diaminobutano, conhecida como putrescina (GARAI et al., 2007). Estas aminas podem apresentar efeitos tóxicos se ingeridas em altas concentrações ou quando o processo natural de desintoxicação do organismo é inibido pela ação de medicamentos ou por alguma característica genética (GARAI et al., 2007; CALZADA, 2013).

Genes de resistência a antibióticos também têm sido encontrados em diversas espécies de BAL, e se tornaram uma preocupação adicional para o uso desses micro-organismos em processos industriais. A resistência aos antibióticos pode ser inerente à espécie ou ao gênero (intrínseca ou natural), ou adquirida através de DNA exógeno ou por mutação genética (BORIES et al., 2008). A segurança dos micro-organismos é mais comprometida quando a resistência é adquirida. Neste caso, os genes de resistência podem ser também transferidos para outras bactérias (ADIMPONG et al., 2012; ZHANG et al., 2013), principalmente aqueles localizados em transposons ou plasmídeos, que podem ser facilmente transferidos para bactérias patogênicas (AYMERICH et al., 2006; DEVIRGILIIS et al., 2011).

Além desses, a presença de outros fatores relacionados às atividades metabólicas das BAL podem ter efeito nocivo à saúde humana, tais como: degradação do ácido hialurônico, atividade de desconjugação de sais biliares, agregação plaquetária, atividade hemolítica e enzimática, tais como a atividade de azoredutase, nitroredutase, *β-glucuronidase*, *β-glucosaminidase* e *β-glucosidase*, além de produção de metabólitos tóxicos (BERNARDEAU et al., 2008; GIRAFFA, 2012).

2.2. Propriedades tecnológicas das BAL

Desde os primeiros estudos sobre a atuação das BAL, cresce sustentavelmente a produção de produtos lácteos fermentados, em especial os queijos. Com relação à fisiologia, as BAL são responsáveis pela fermentação e produção de ácidos, tornando sua aplicação cada vez mais importante em produtos lácteos (BROADBENT; STEELE, 2005; CHERIGUENE et al., 2007; DE DEA LINDNER, 2008). Além disso, a biodiversidade das BAL envolvidas na produção de queijos e de outros derivados lácteos pode ser considerada um fator fundamental para a manutenção das características típicas de sabor e aroma dos produtos (ERCOLINI et al., 2001b; MARINO, MAIFRENI; RONDININI, 2003; GASPAR et al., 2013). Assim, a produção de queijos de alta qualidade requer uma estreita atenção para a caracterização (POGACIC et al., 2013), diferenciação e manutenção das cepas de BAL envolvidas no processo produtivo (GIRAFFA et al., 2003).

O grupo dos lactobacilos é bem representativo sobre os outros presentes em leite e seus derivados, e dentre as BAL, é considerado o mais seguro (SALVETTI; TORRIANI; FELIS, 2012). Algumas espécies apresentam propriedades fisiológicas para produção intensa de ácido durante a fermentação, sendo os principais responsáveis pela redução do pH e, consequentemente, pela coagulação do leite (coagulação ácida).

Além dos ácidos orgânicos (como ácido lático), existem vários outros metabólitos produzidos por BAL de interesse tecnológico, tais como peróxido de hidrogênio, dióxido de carbono, diacetil, acetaldeído e substâncias antimicrobianas de natureza protéica, como as bacteriocinas (THARMARAJ; SHAH, 2009; REIS et al., 2012; GASPAR, et al., 2013; DE PAULA et al., 2014). Algumas BAL também podem ser importantes nos alimentos pela sua função probiótica (SUSKOVIC et al., 2010; REIS et al., 2011; AHMADOVA, et al., 2013; JERONYMO-CENEVIVA et al., 2014). Assim, quando apresentam características como probióticas e são administradas em quantidade adequada, conferem benefícios à saúde do consumidor (FAO/WHO, 2002), como o equilíbrio da microbiota intestinal, aumento da resposta imune, redução da intolerância à lactose, inibição de micro-organismos patogênicos pela produção de compostos naturais, entre outros. Esses micro-organismos também produzem grande número de enzimas glicolíticas, lipolíticas e proteolíticas, as quais transformam os nutrientes fundamentais do leite e dos seus derivados em compostos com propriedades sensoriais desejáveis (VILJOEN, 2001; PENNA et al., 2015).

Além da ação microbiana enzimática, diretamente relacionada às reações proteolíticas, a percepção sensorial dos produtos lácteos, por ser um processo complexo, é influenciada por muitos fatores, tais como a composição de compostos flavorizantes, a textura e a aparência do produto (SMIT; SMIT; ENGELS, 2005). Assim, a degradação da caseína resultante da ação das proteases (extra ou intracelular) e peptidases produzidas por BAL, também contribui para a formação da textura e características sensoriais dos queijos. Adicionalmente, os aminoácidos formados podem ainda ser degradados em aminas, ésteres, ácidos e tióis, contribuindo para a produção de diferentes aromas (PRIETO et al., 2002).

Estudos demonstram ainda, a habilidade das culturas autóctones de BAL em produzir aromas distintos, comparados com aqueles produzidos por culturas comerciais. Provavelmente, isso se dá devido à capacidade destas culturas em produzir maiores quantidades de enzimas conversoras de aminoácidos precursores de compostos aromáticos específicos (CENTENO; CEPADA; RODRIGUEZ-OTERO, 1996).

2.2.1. Metabolismo do citrato

O citrato é abundante na natureza e no leite, e é um constituinte natural de todas as células vivas, sendo uma importante fonte de energia para algumas BAL, sob condições de aerobiose e anaerobiose; entretanto, um número limitado de BAL é capaz de cataboliza-lo (DRIDER et al., 2004).

A habilidade de metabolizar o citrato é dependente da presença da enzima citrato permease (CitP), que é bem descrita para algumas espécies e linhagens de *Lactococcus* sp., *Lactobacillus* sp. e *Enterococcus* sp., bem como para linhagens pertencentes aos gêneros *Leuconostoc* e *Oenococcus* (BANDELL et al., 1998).

O citrato pode ser utilizado como única fonte de energia ou ser co-metabolizado. Para BAL heterofermentativas como *Leuconostoc mesenteroides* e *Lactobacillus fermentum*, o citrato é convertido em piruvato, que então é reduzido a lactato. Nesse caso, quando o citrato está presente, mais energia é gerada durante o metabolismo do açúcar (SCHMITT et al., 1992). Particularmente, para *Lactobacillus casei* (Lactobacilos heterofermentativo facultativo), algumas linhagens são capazes de utilizar o citrato quando a glicose é limitada. Para as BAL homofermentativas, tais como *L. lactis* subsp. *lactis* biovar. *diacetylactis* e *Enterococcus* sp., o piruvato é o intermediário comum formado durante o metabolismo do açúcar e do citrato (VANINGELGEM et al., 2006).

Quando o citrato está presente como fonte de energia exclusiva, a sua absorção pela célula bacteriana ocorre via simporte de citrato divalente e um próton ou via uniporte de citrato monovalente (KONINGS, 2002). Durante a cometabolismo do citrato e um açúcar (fermentação citrolática), CitP catalisa a troca (antiporte) de citrato divalente aniônico e lactato monovalente (HUGENHOLTZ, 1993). A absorção do citrato é um processo eletrogênico, que em conjunto com a formação de um gradiente de pH através da membrana celular, resulta na formação de uma força próton motiva e, consequentemente, a geração de energia metabólica (BANDELL et al., 1998). Após o transporte para dentro da célula, o citrato é convertido em oxaloacetato e acetato pela enzima citrato liase (CitL). O oxaloacetato pode ser ainda convertido pela descarboxilase oxaloacetato (CitM), obtendo-se o piruvato e dióxido de carbono (HUGENHOLTZ, 1993).

O subsequente metabolismo do piruvato leva a produção de compostos aromáticos, tais como: acetaldeído, acetoina, 2,3-butanadiol e diacetil (SMID; KLEEREBEZEM, 2014). Além disso, o cometabolismo da lactose e do citrato pode levar a produção de altas concentrações de etanol, ácido acético e outros compostos responsáveis pelo sabor de queijos moles e leites fermentados (McSWEENEY; SOUSA, 2000; MILESI et al., 2010). O dióxido de carbono também produzido a partir do metabolismo do citrato pode ser importante para a formação de olhaduras, que contribuem para a textura e características peculiares de alguns tipos de queijos, como os queijos azuis (DE FIGUEROA et al., 2001; VANINGELGEM et al., 2006). A Figura 2 representa as principais rotas bioquímicas do metabolismo do piruvato pelas BAL capazes de utilizar o citrato.

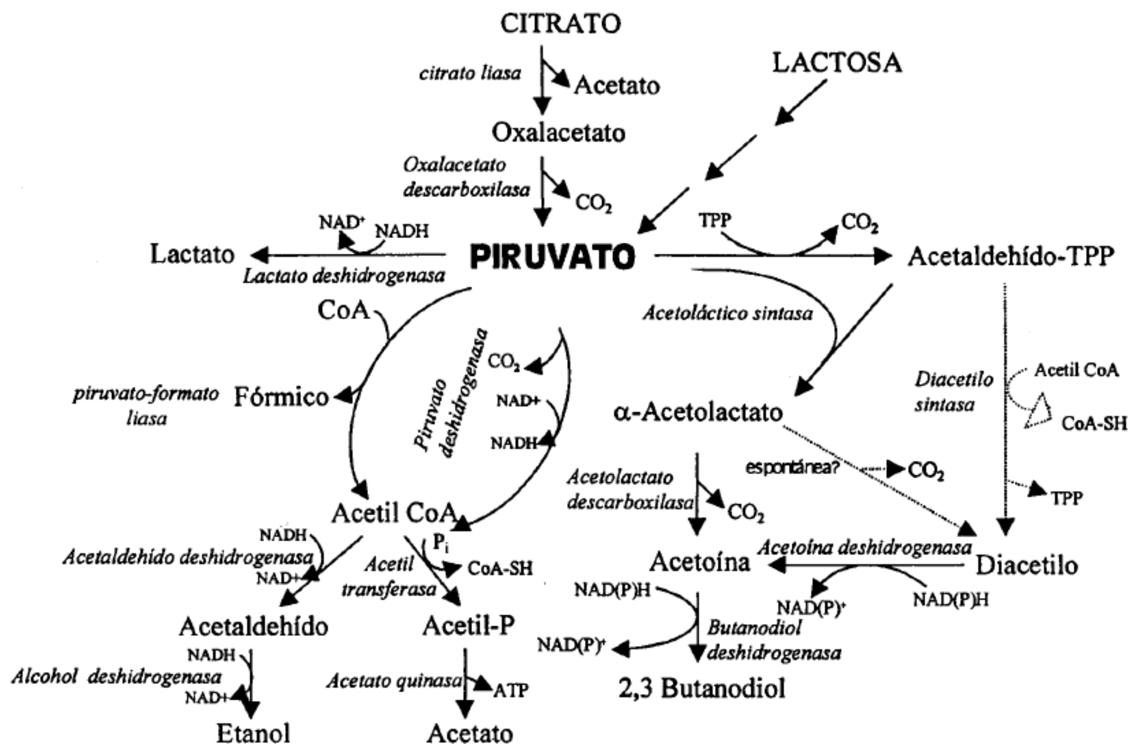

Figura 2- Rotas bioquímicas do metabolismo do piruvato pelas BAL capazes de utilizar citrato.

2.2.2. Produção de diacetil e acetoina

Diacetil (2,3-butanodiona) e acetoína (3-hydroxy-2-butanona) são dois importantes compostos carbonílicos voláteis produzidos por BAL a partir do metabolismo do citrato (AUNSBJERG et al., 2015). Pesquisas relatam que culturas termofílicas usadas para a produção de queijos (PAPPA et al., 2013) e iogurtes (BESHKOVA et al., 2003) produzem maiores concentrações de diacetil em relação às culturas mesofílicas.

O diacetil é comumente presente em muitos produtos lácteos fermentados aos quais confere cremosidade e aroma amanteigado. Geralmente está presente em altas concentrações nos iogurtes e nas manteigas, e é essencial para a produção de queijos como Camembert, Cheddar e Emmental (CURIONI; BOSSET, 2002). Adicionalmente, este composto tem sido associado às propriedades antifúngicas e antibacterianas nos diversos processos fermentativos (JAY, 2000; AUNSBJERG et al., 2015). Este composto é um subproduto do metabolismo do piruvato, que pode ser facilmente convertido em acetoína pela enzima acetoína redutase (CRUZ et al., 2012). A acetoína também pode ser produzida a partir da redução do acetaldeído pela enzima α-acetolactato descarboxilase (Figura 3). O aroma da acetoína e suas

propriedades são semelhantes ao do diacetil, no entanto, é consideravelmente mais fraco, o que tende a reduzir a intensidade do diacetil, quando presente. A acetoína tem sabor mais leve, agradável e suave (CHENG, 2010).

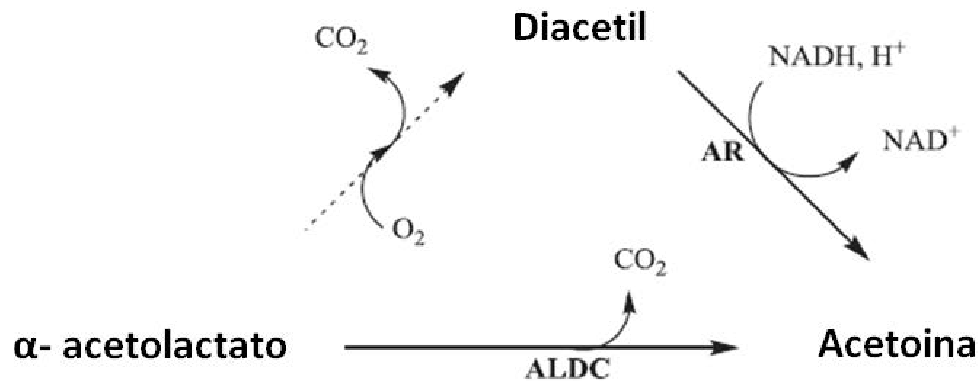

Figura 3 - Rota bioquímica da produção de acetoína a partir do diacetil e do α-acetolactato pelas bactérias acidoláticas (Adaptado de VONWRIGHT; AXELSSON, 2011). ALDC: α-acetolactato descarboxilase; AR: acetoina redutase.

2.2.3. Produção de ácidos orgânicos por BAL

Os processos fermentativos são caracterizados principalmente pela produção e acumulo dos ácidos orgânicos produzidos pelas BAL, que consequentemente reduz os valores de pH do meio (LEITE et al., 2013). Assim, além de contribuir para as características sensoriais dos produtos lácteos, os ácidos orgânicos são importantes para o processo tecnológico e para biopreservação do produto. Por exemplo, durante a produção dos leites fermentados, a redução do pH do leite é importante para a precipitação da caseína, imprescindível para a formação do gel. No caso do queijo Muçarela, o baixo pH da coalhada permite o esticamento da massa em água quente, resultando em uma massa coesa, característica deste queijo (DE ANGELIS et al., 2008). Quanto à sua relação com a biopreservação do produto, o baixo valor de pH também tem ação bacteriostática contra micro-organismos patogênicos, resultando em vida útil prolongada e maior segurança ao produto (PENNA et al., 2015).

A produção dos ácidos orgânicos ocorre durante a fermentação da lactose pelas BAL. Estes ácidos orgânicos também estão presentes nos produtos lácteos como resultado da

hidrólise da gordura presente no leite (ácidos graxos livres, tais como ácido acético e butírico), produção ou adição direta como acidulante (ácido lático e cítrico), metabolismo bioquímico (ácidos úrico, cítrico e orótico), e/ou relacionados com o crescimento do micro-organismo (ácidos lático, acético, pirúvico, e fórmico) (IZCO et al., 2002).

De acordo com Chandan and Kilara (2013), os ácidos lático e acético são os principais ácidos produzidos a partir do metabolismo da glicose, enquanto os outros ácidos (pirúvico, propiônico, fórmico, orótico, cítrico e butírico) são produzidos em menores quantidades.

O ácido lático tem sido utilizado tradicionalmente nos produtos de lacticínios como conservante e aditivo de sabor (GASPAR et al., 2013). Este é um ácido não volátil, que apresenta sabor suave e agradável ao paladar quando presente em concentrações adequadas (CARVALHO et al., 2005). Dois tipos de ácido lático L (+) e D (-) são produzidos pelas BAL. Algumas espécies de bactérias, incluindo *Lb. bulgaricus* e *L. lactis* produzem apenas D (-) ácido lático, enquanto *St. thermphilus* e *Lb. casei* produzem L (+) ácido lático. *Lb. helveticus* e *Lactobacillus acidophilus*, por exemplo, são capazes de produzir os dois tipos (CHANDAN; KILARA, 2013). Nutricionalmente, o tipo D (-) é indesejável uma vez que a sua forma isomérica não é facilmente metabolizada por mamíferos, incluindo os seres humanos (LIU, 2003).

O ácido acético é produzido principalmente por BAL heterofermentativas por meio da oxidação do acetaldeído, que por sua vez é produzido a partir da hidratação do acetileno ou por desidrogenação catalítica do etanol (BAMFORTH, 2005). Algumas espécies de BAL, principalmente alguns *Lactobacillus*, também podem produzir ácido acético a partir do metabolismo do citrato (ØSTLIE; HELLAND; NARVHUS, 2003). Nos produtos fermentados, esse ácido é responsável pelo sabor de vinagre, e muitas vezes, é produzido por BAL, tais como *Lb. rhamnosus* (OLIVEIRA et al., 2012), *L. lactis* subsp. *lactis* e *L. lactis* subsp. *cremoris* (SALMINEN; VON WRIGHT; OUWEHAND, 2004). Acredita-se que o ácido acético tem um efeito sinérgico com o ácido lático na prevenção do crescimento de fungos (DANG et al., 2009). Recentemente, estudos demonstraram a ação do ácido lático e acético como principais substâncias antifúngicas produzidas por *Leuconostoc citreum* e *Weissella confusa* (BAEK et al., 2012), e em concentrações superiores a 17,5 mM, estes ácidos orgânicos foram apontados como responsáveis por retardar o desenvolvimento de *Cladosporium* sp. YS1 e *Penicillium crustosum* YS2 (CROWLEY; MAHONY; SINDEREN, 2013).

Os outros ácidos produzidos pelas BAL em menores concentrações também são muito utilizados na indústria de alimentos, apresentando grande importância para este setor. O ácido cítrico, por sua vez, tem sabor agradável, apresenta fácil assimilação pelo organismo humano e baixa toxicidade. Devido a estas características, este ácido é amplamente utilizado como agente acidulante para intensificar o sabor e a estabilidade dos produtos lácteos (IZCO et al., 2002). O efeito antibacteriano do ácido cítrico não está ligado apenas à sua ação ácida, mas também à sua atividade quelante dos íons de Ca^{2+} (GRAHAM; LUND, 1986).

O ácido orótico, também conhecido como vitamina B13, normalmente está associado ao crescimento das BAL (FERNANDEZ-GARCIA; MCGREGOR, 1994). Este ácido é um agente intermediário da síntese de ácidos nucleicos, e acredita-se ter um efeito hipocolesterolêmico, sendo encontrado em produtos lácteos probióticos (ALHAJ et al., 2007). Adicionalmente, o ácido orótico tem demonstrado ações nootrópicas em estudos com jovens e idosos com deficiências cognitivas, e tem sido amplamente utilizado para tratamentos neurológicos ou para correção de déficits metabólicos (GAY et al., 2011).

A produção de ácido fórmico e pirúvico pelas BAL é importante no processo fermentativo do iogurte, em que *Lb. bulgaricus* e *St. thermophilus* exibem uma relação simbiótica. Neste caso, *St. thermophilus* produz ácido fórmico e ácido pirúvico para o crescimento do *Lb. bulgaricus*. De acordo com Suzuki et al. (1986), o ácido fórmico produzido por *St. thermophilus* é utilizado como um precursor da síntese de purina para a síntese das células de *Lb. bulgaricus*. *Lb. bulgaricus*, por sua vez, apresenta um sistema proteolítico mais eficiente e fornece peptídos e/ou aminoácidos essenciais para o desenvolvimento de *St. thermophilus* (NISHIMURA et al., 2013). Adicionalmente, altas concentrações de ácido fórmico produzidas por BAL podem contribuir para a inibição de *Listeria* sp. em produtos lácteos (AFZAL et al., 2010). O ácido pirúvico, por sua vez, pode ser utilizado no setor industrial como antioxidante e termogênico, e também para a produção de polímeros e cosméticos (AARNIKUNNAS et al., 2003).

2.3. BAL envolvidas na produção do queijo Muçarela

A presença de BAL no queijo Muçarela de búfala imerso em soro foi investigada por alguns autores (COPPOLA et al., 1988; COPPOLA et al., 1990; PARENTE et al., 1997; MOREA; BARUZZI; COCCONCELLI, 1999), porém, com poucas informações sobre a microbiota lática envolvida. Estudos mais recentes mostram que cepas de *Lactobacillus* sp.

pertencentes às espécies *Lb helveticus, Lb lactis, Lb. bulgaricus* e *Lb. fermentum e* cocos, como *St. thermophilus, Lactococcus* sp.*, Lc. mesenteroides*, assim como *Enterococcus* sp. têm sido relatadas nesse queijo (ERCOLINI et al., 2001b; ERCOLINI et al., 2004; ERCOLINI et al., 2012; SILVA et al., 2015).

As culturas láticas, comuns em queijo Muçarela de búfala, podem ser classificadas em: bactérias acidoláticas *starter* (*Starter Lactic Acid Bacteria* - SLAB) ou iniciadoras, quando são as principais responsáveis por metabolizar a lactose e não *starter* (*Non Starter Lactic Acid Bacteria* - NSLAB), se presentes durante o processo de fabricação e período de estocagem. Segundo De Dea Lindner (2008), SLAB mesófilas como *L. lactis* e *L. lactis* subsp. *cremoris*, e termófilas como *St. thermophilus, Lb. helveticus* e *Lb. bulgaricus* são dominantes em culturas usadas na elaboração de queijo Muçarela. Parente, Moschetti e Coppola (1998) e Ercolini e colaboradores (2012) também relataram a presença dessas espécies no soro fermento (NWC - *natural whey culture*) empregado na elaboração do queijo Muçarela.

As culturas não *starter* (*Non Starter Lactic Acid Bacteria* - NSLAB), geralmente, são originadas do leite *in natura*, do ambiente de produção e, em quantidade minoritária, do soro fermento natural (SAVIJOKI; INGMER; VARMANEN, 2006). As NSLAB têm como fonte energética alternativa os aminoácidos liberados da autólise das SLAB, assim como, os resíduos de carboidratos liberados de glicoproteínas e glicolipídeos presentes no leite (WILLIAMS; WITHERS; BANKS, 2000).

Em diferentes variedades de queijos elaborados com leite *in natura* ou pasteurizado, os lactobacilos heterofermentativos facultativos são a classe dominante de NSLB, especialmente *Lb. casei, Lactobacillus plantarum* e *Lactobacillus curvatus.* Os lactobacilos estritos heterofermentativos, particularmente *Lactobacillus brevis*, são encontrados em menor quantidade (BERESFORD; WILLIAMS, 2004; BROADBENT; STEELE, 2005).

As culturas *starters* podem ser compostas por dois tipos de bactérias: as produtoras de ácidos e as produtoras de aromas. Dessa forma, os ácidos podem ser produzidos por *St. thermophilus*, *Lb. bulgaricus, L. lactis* subsp. *cremoris* e por *L. lactis* subsp. *lactis,* enquanto o aroma é produzido por *L. lactis* subsp. *lactis* biovar. *diacetylactis* e por espécies do gênero *Leuconostoc.*

Os lactobacilos homofermentativos obrigatórios, como *Lb. helveticus* e *Lb. bulgaricus,* são geralmente usados como culturas *starters* e/ou isolados do processo de produção de diversos queijos duros ou de massa filada, como o queijo Muçarela de búfala (ERCOLINI et al., 2004; ERCOLINI et al., 2012; SILVA et al., 2015). Culturas termofílicas puras de *St.*

thermophilus ou mista de *St. thermophilus* e *Lb. helveticus* ou *Lb. bulgaricus* são frequentemente utilizadas como culturas comerciais *starters* para a fabricação de diversos queijos e de leite fermentado (DE CANDIA et al., 2007) e têm sido isoladas de soro fermento utilizado na elaboração de queijos de massa filada, tal como a Muçarela de búfala (ERCOLINI et al., 2012).

St. thermophilus é uma BAL homofermentativa, bastante usada em fermentações láticas como culturas *starters*, devido à sua capacidade de produzir altas concentrações de ácido lático pelo catabolismo da lactose (GALIA et al., 2009), e também por ser capaz de produzir altos teores de compostos aromáticos em queijos (REPIZO et al., 2013). Esta espécie é a principal BAL utilizada para a produção do queijo Muçarela como cultura *starter*, pura ou em co-cultura com outras BAL, onde é responsável pela rápida acidificação da coalhada e capaz de sobreviver às altas temperaturas requeridas durante o processo de produção desse queijo (DE ANGELIS et al., 2008).

Espécies pertencentes ao gênero *Leuconostoc* também têm sido isoladas de queijo Muçarela de búfala (SILVA et al., 2015) e presentes em diversos processos fermentativos. Este gênero se distingue das outras BAL por serem cocos mesófilos heterofermentativos (MARTH; STEELE, 2001). Esta BAL é capaz de produzir CO_2, d-lactato, etanol ou acetato, a partir do metabolismo da glicose. Entretanto, este micro-organismo geralmente é utilizado em produtos lácteos por sua habilidade em produzir compostos aromáticos a partir do citrato (SAVIJOKI; INGMER; VARMANEN, 2006). Além disso, *Leuconostoc* sp. é comumente encontrado em queijos fabricados com leite *in natura* (CALLON; MILLET; MONTEL, 2004; HEMME; FOUCAUD-SCHEUNEMANN, 2004).

Enterococcus sp. também tem sido isolado de queijo Muçarela (SILVA et al., 2015) e é alvo de diversas pesquisas que buscam avaliar a sua funcionalidade tecnológica nos queijos (GIRAFFA et al., 2003; GOMES et al., 2008; MARTY et al., 2012). Esses micro-organismos são cocos homofermentativos capazes de crescer em temperaturas extremas e são encontrados em diversas fontes alimentícias. *Enterococcus* sp. desempenham um papel reconhecido no desenvolvimento das características sensoriais durante a fabricação e maturação de alguns queijos (SARANTINOPOULOS; KALANTZOPOULOS; TSAKALIDOU, 2002), além de produzirem altas concentrações de ácido lático e de serem capazes de metabolizar o citrato. Estirpes de *E. faecium*, *E. faecalis* e *E. durans* em combinação com outras espécies de BAL foram utilizados como parte de culturas *starters* para a fabricação do queijo Muçarela de

búfala e influenciaram positivamente as caracteristicas sensoriais do produto (DE ANGELIS et al., 2008), no entanto, a segurança dos *Enterococcus* nos alimentos ainda é contestada.

Culturas mesófilicas de *Lactococcus* sp. também são usadas na elaboração de uma variedade de produtos lácteos, incluindo queijos maturados e não-maturados (SAMARZIJA et al., 2001). Este micro-organismo foi dominante em leite de búfala *in natura* utilizado na fabricação do queijo Muçarela (ERCOLINI et al., 2012). A qualidade de alguns produtos fermentados é resultante da ação de *L. lactis* subsp. *lactis* biovar. *diacetylactis,* entre outras BAL, capazes de produzir compostos aromatizantes como a acetoína e o diacetil pela utilização de citrato (COGAN, 1995; DE FIGUEROA et al., 2001; REPIZO et al., 2013) (Figura 2). Entretanto, a produção desses compostos em queijo Muçarela ainda não foi evidenciada.

3. Isolamento e identificação de BAL

Um dos principais interesses dos microbiologistas que atuam na área de produtos lácteos fermentados é estudar a diversidade e a dinâmica dos micro-organismos durante a fabricação dos produtos e seu período de estocagem, bem como correlacionar a ocorrência de determinadas espécies de bactérias ou linhagens com a presença de características sensoriais específicas no produto final.

Tradicionalmente, o conhecimento da diversidade bacteriana em queijos é resultado do estudo das culturas a partir do crescimento dos micro-organismos em meios seletivos e sua posterior identificação em gênero e/ou espécie, com base nas características fenotípicas e fisiológicas (RANDAZZO; CAGGIA; NEVIANI, 2009).

Para o isolamento de micro-organismos, em particular as BAL, usa-se meios seletivos e condições adequadas para o seu crescimento. No caso do *Lactobacillus* sp. geralmente usa-se o meio MRS (De Man, Rogosa, Sharpe) acidificado até pH 5,4 (IDF, 1997), em condições de anaerobiose ou microaerofilia e incubação entre 25-30 °C ou a 42-45 °C, para o isolamento de mesófilos e termófilos, respectivamente. No entanto, para os cocos, geralmente se usa o meio de cultura M17, com modificações ou não, e incubação nas mesmas condições (DE DEA LINDNER, 2008; GATTI et al., 2008; SILVA, 2010). Um impasse encontrado para a caracterização da microbiota lática presente nas matrizes alimentícias é que muitas BAL, entre elas as NSLAB, podem estar presentes no alimento em condições vitais, porém não cultiváveis (GATTI et al., 2008; ACHILLEOS; BERTHIER, 2013). Por esta razão, em alguns

casos, em que a identificação da microbiota é necessária para melhor compreender certas situações tecnológicas, um meio de cultivo nutricionalmente adequado, e se possível, de composição análoga à matriz de isolamento, seria ideal para recuperar esta microbiota (DE DEA LINDNER, 2008; NEVIANI et al., 2009).

O estudo morfológico de BAL deve considerar tanto as características da colônia (cor, tamanho, forma, textura) quanto celular (forma da célula, ausência de endósporos) (DE DEA LINDNER, 2008). A descrição dos caracteres fisiológicos e bioquímicos implica em informações sobre suas propriedades, tais como: desenvolvimento em diferentes temperaturas, valores de pH, concentrações de sal, capacidade de assimilar O_2 e de produzir CO_2, desenvolvimento na presença de diversas substâncias, incluindo anti-microbianos, viabilidade na presença de diversas enzimas, utilização de compostos orgânicos e, principalmente, capacidade fermentativa (KRIEG; HOLT, 1984). Porém, a identificação com base na morfologia e a descrição dos caracteres fisiológicos apresenta dificuldade quando na utilização de técnicas microbiológicas tradicionais (WALTERS et al., 2000). A identificação das BAL baseada em testes bioquímicos, ou o uso de kits rápidos, também é difícil, podendo apresentar falsos resultados, ou mesmo não conseguir identificar a espécie (CHERIGUENE et al., 2007).

Ao contrário dos métodos fenotípicos e fisiológicos, a identificação por técnicas moleculares são muito mais consistentes, rápidas, confiáveis e reprodutíveis, podendo discriminar espécies que estão em grupos estreitamente indistinguíveis quando identificados por testes tradicionais. Além disso, muitas espécies de *Lactobacillus*, entre outros, foram reclassificadas usando técnicas moleculares baseadas na reação em cadeia da polimerase (PCR - *Polimerase Chain Reaction*) (DELLAGLIO; TORRIANI; FELIS, 2004).

Técnicas com base no sequenciamento do DNA e de regiões conservadas do RNA têm sido muito utilizadas para a identificação de BAL. Além disso, técnicas como *Restriction Fragment Length Polymorphism* (RFLP), que usa enzimas de restrição específicas e resulta em fragmentos genômicos, também têm sido usadas para identificação de BAL (MANCINI et al., 2012). Da mesma forma, a técnica de *Randomly Amplified Polymorphic* DNA (RAPD) também tem sido aplicada extensivamente para a diferenciação intra-específica de BAL isoladas de alimentos fermentados (RANDAZZO; CAGGIA; NEVIANI, 2009; MANCINI et al., 2012; APONTE et al., 2013).

3.1. Identificação e biotipagem de BAL pelo sequenciamento do gene 16S rRNA e pelas técnicas de RAPD e RFLP

Atualmente, a maioria das técnicas moleculares baseadas em genes ribossomais utilizadas extensivamente para a identificação de BAL está bem consolidada, sendo aplicadas para a caracterização e identificação da microbiota lática presente nos diversos produtos lácteos (RANDAZZO; CAGGIA; NEVIANI, 2009). Os genes ribossomais são mais conservados que a maioria dos genes do genoma, e dificilmente são afetados pela pressão ambiental, sendo bem conservados e comuns em várias espécies (MOHANIA et al., 2008). Assim, o sequenciamento da região 16S rRNA presente em procariotos, contém informações taxonômicas superiores quando comparadas aos dados produzidos por híbridos de DNA-DNA (BOTINA; TSYGANKOV; SUKHODOLETS, 2006), e é indiscutivelmente, o método mais usado para a identificação de BAL em queijos (MOREA et al., 1998; BARUZZI et al., 2002; AQUILANTI et al., 2007; DOLCI et al., 2008; AKABANDA et al., 2013; DELGADO et al., 2013).

A técnica de sequenciamento do gene 16S rRNA, juntamente com técnicas de biotipagem baseadas no DNA, são utilizadas para a identificação de espécies da microbiota presente em derivados lácteos. Assim, micro-organismos dos gêneros *Lactobacillus, Lactococcus, Leuconostoc, Enterococcus, Pediococcus e Streptococcus* foram identificados nesses produtos (OGIER et al., 2002). Com base no sequênciamento do gene 16S rRNA foi possível diferenciar a cepa de *Lb. delbrueckii* subsp. *lactis* que, constantemente, é confundida com o perfil fermentativo de *Lb. helveticus*, quando se utiliza metodologias tradicionais (HEBERT et al., 2000). Da mesma forma, as espécies *Lb. helveticus, Lb. plantarum, Lb. casei e Lb. fermentum* foram identificados em soro fermento utilizado para a fabricação do queijo Muçarela (DE CANDIA et al., 2007), e *St. thermophillus*, *Lb. bulgaricus* e *Lc. mesenteroides* foram isolados de queijo Muçarela de búfala durante o período de estocagem (SILVA et al., 2015).

A tipagem das BAL usando diferentes técnicas moleculares, aliadas ou não as técnicas tradicionais, tem sido usada com sucesso para a identificação dessas culturas (APONTE et al., 2013). Dentre os métodos dependentes de cultivo, a técnica de RAPD é a mais utilizada para a caracterização da biota lática presente em queijos (MANCINI et al., 2012), bem como a aplicação de RFLP da região intergênica (ITS) 16S - 23S rDNA, cujos resultados são

adequados para esta finalidade (BEN BELGACEM et al., 2009; MANCINI et al., 2012; POGACIC et al., 2013).

A técnica de RAPD não requer o conhecimento prévio do DNA a ser analisado. Nesta técnica, utiliza-se *primers* inespecíficos de até 10 pares de base, com sequência arbitrária, para a realização da PCR (DE CANDIA et al., 2007; GATTI et al., 2008), gerando uma matriz de *amplicon*s de DNA anônimo (BEN AMOR; VAUGHAN; DE VOS, 2007). Os produtos da PCR são submetidos à separação por eletroforese, onde se obtêm padrões de DNAs. As características visuais das bandas resultantes dos fragmentos permitem presumir o grau de parentesco entre os DNAs analisados (VALÉRIO; WEIKERT-OLIVEIRA; RESENDE, 2006).

Apesar da técnica de RAPD ser um método simples e rápido, a sua reprodutibilidade, otimização e padronização apresentam dificuldades. Diferenças entre termocicladores, reagentes, *Taq* polimerase e qualidade do DNA podem causar variações nos padrões de RAPD, e consequentemente, os padrões obtidos em diferentes laboratórios nem sempre são comparáveis (RANDAZZO; CAGGIA; NEVIANI, 2009; MANCINI et al., 2012).

A despeito das limitações, esta técnica é amplamente utilizada para a caracterização dos micro-organismos isolados da matriz dos queijos, permitindo a diferenciação entre as espécies e, em alguns casos, entre as linhagens da mesma espécie. Desta forma, a análise de RAPD é considerada um método confiável para discernir entre as espécies *starter* e não *starter* presentes no queijo, ou mesmo, monitorar mudanças na comunidade de BAL durante o processo de fermentação (RANDAZZO; CAGGIA; NEVIANI, 2009).

Protocolos de RAPD *fingerprints* têm sido desenvolvidos para a identificação de BAL advindas de produtos lácteos, picles e da microbiota gastrintestinal (DOLCI et al., 2008; PLATERO et al., 2009) e para cepas *St. thermophilus* e *Lactobacillus* sp. isoladas de amostras de queijo Muçarela (CANCILLA et al., 1992; LANGA; FERNANDEZ; MARTIN, 2003; DE CANDIA et al., 2007; SILVA et al., 2015).

A sequência do gene ribossomal DNA (rDNA) e da região intergênica é também usada para determinar as relações filogenéticas dos micro-organismos presentes em alimentos fermentados (JEYARAM et al., 2010; MARKIEWICZ et al., 2010) e em produtos lácteos (HOPPE-SEYLER et al., 2007). A região intergênica apresenta variações em seu tamanho e em sua sequencia, dependendo da presença ou ausência de genes específicos tRNA. Assim, a análise da região $tRNA^{Ala}$ - 23 rDNA por RFLP-PCR (*Restriction Fragment Length*

Polymorphism) tem sido utilizada para a identificação de BAL isoladas de derivados cárneos (BEN BELGACEM et al., 2009) e de produtos lácteos (MANCINI et al., 2012).

A técnica de RFLP, utilizada para determinar as relações filogenéticas entre as BAL, é considerada mais reprodutiva que RAPD. Nesse caso, os micro-organismos podem ser diferenciados pelo perfil de fragmentos obtidos por ação de enzimas de restrição específicas (endonucleases). Desta forma, as espécies são identificadas comparando as variações nas sequencias dos DNAs resultantes da ação das enzimas de restrição e pela variação no tamanho e número de fragmentos obtidos por ação dessas enzimas (DE DEA LINDNER, 2008). As endonucleases de restrição clivam o DNA em sítios com sequencias específicas de reconhecimento, cuja posição no genoma é característica de cada espécie ou subespécie. Para a visualização dos *amplicons,* os fragmentos são separados por eletroforese em gel de agarose ou poliacrilamida, sob a influência de um campo elétrico unidirecional (BOTELHO, 2005).

Utilizando a técnica de RFLP, cepas isoladas de produtos fermentados e de derivados lácteos, como *L. lactis*, *Enterococcus faecium*, *Enterococcus faecalis*, *Lactobacillus sakei*, *Lactobacillus curvatus*, *Lactobacillus plantarum*, *Lactobacillus alimentarius*, *Pediococcus pentosaceus*, *Weisella confusa* (BEN BELGACEM et al., 2009) e *Lb. delbrueckii* (GIRAFFA et al., 2003) foram identificadas.

Embora essas técnicas moleculares sejam muito utilizadas para a avaliação da diversidade microbiana dos queijos (RANDAZZO; CAGGIA; NEVIANI, 2009), o uso de tais técnicas apresenta limitações para avaliar a microbiota total, principalmente para avaliar a população não cultivável. Por outro lado, as técnicas independentes de cultivo apresentam melhores resultados para a estimativa da população total de BAL no produto (POGACICI et al., 2013).

3.2. Métodos independentes de cultivo frequentemente utilizados para a identificação de BAL

Atualmente, técnicas independentes de cultivo são amplamente usadas para a identificação de BAL, nas quais o DNA genômico bacteriano e/ou RNA deve ser extraído diretamente da amostra, seguido pela amplificação das regiões variáveis do gene 16S ou outros genes conservados. Se o DNA total da microbiota é usado na PCR, a técnica pode fornecer um perfil da diversidade genética, enquanto que, se o RNA total é usado, o perfil indica a microbiota metabolicamente ativa (RANTSIOU et al., 2008; ACHILLEOS;

BERTHIER, 2013). Estes métodos são amplamente utilizados para caracterizar microbiologicamente alimentos fermentados, incluindo derivados lácteos, uma vez que uma população minoritária de BAL presente na matriz pode não ser visualizada, ou mesmo não ser cultivável por métodos tradicionais (ERCOLINI et al., 2001a; GATTI et al., 2008). Assim, métodos independentes de cultivo tornam-se viáveis para compreender melhor a ecologia destes micro-organismos. Para a caracterização da comunidade de BAL, as técnicas de *PCR-Denaturing Gradient Gel Electrophoresis* (DGGE) e *PCR-Temperature Gradient Gel Electrophoresis* (TGGE) são mais utilizadas (POGACICI et al., 2010).

A população microbiana pode ser identificada por DGGE ou TGGE por comparação da posição da banda da cepa de interesse com a da cepa de referência, ou por sequenciamento. Para a PCR-DGGE, a desnaturação é feita com desnaturantes químicos (formamida e ureia), incorporadas ao gel de acrilamida, com um gradiente de desnaturação linear à temperatura constante, entre 55 e 65°C. Para PCR-TGGE, o gradiente de desnaturação é obtido pela variação da temperatura ao longo do tempo, sem o uso de produtos químicos (JANY; BARBIER, 2008). As principais vantagens das técnicas DGGE e TGGE são o monitoramento das mudanças da comunidade microbiana e uma visão simples das espécies de BAL presentes em uma amostra (POGACIC et al., 2010).

A técnica de FISH (*Fluorescence in situ hybridization*) também tem sido utilizada para a identificação de BAL. Nesta técnica utilizam-se sondas fluorescentes marcadas, que se ligam às sequencias do cromossomo que apresentam alto grau de similaridade. É usada para verificar a distribuição espacial dos micro-organismos (POGACIC et al., 2010).

As técnicas de *Terminal Restriction Fragment Length Polymorphism* (T-RFLP) e *Length Heterogeneity PCR* (LH-PCR) também são muito utilizadas para a identificação de BAL, sendo rápidas e sensíveis. A técnica de T-RFLP é utilizada para avaliar a diversidade de comunidades microbianas complexas, como as dos queijos, baseadas no polimorfismo das regiões do gene 16S rRNA, gerada por enzimas de restrição, enquanto que a técnica LH-PCR, distingue os diferentes micro-organismos com base nas variações naturais no tamanho das sequencias do gene 16S rRNA (RANDAZZO; CAGGIA; NEVIANI, 2009; POGACIC et al., 2010). Esta técnica também tem sido bastante utilizada para a identificação de culturas láticas em queijos (DE DEA LINDNER et al., 2008; SANTARELLI et al., 2013; POGACIC et al., 2013).

A técnica molecular *Quantitative Real Time* – PCR (RealT-qPCR) é cada vez mais aplicada como um método rápido e sensível para a quantificação molecular de bactérias em

produtos lácteos (BOTTARI et al., 2013). Este método usa probes fluorescentes para monitorar a amplificação do DNA alvo em tempo real, permitindo a quantificação da espécie alvo (ZAGO et al., 2009; ACHILLEOS; BERTHIER, 2013). O RealT-qPCR possibilita a detecção do produto da PCR, evitando a necessidade de um pós-processamento, como PCR em gel (agarose, poliacrilamida).

Embora as técnicas independentes de cultivo sejam excelentes para a caracterização da microbiota lática total envolvida nos processos fermentativos, o isolamento e a caracterização morfo-fisiológica das BAL são importantes para compreender as suas propriedades tecnológicas. Além disso, muitas vezes, as técnicas independentes de cultivo apresentam dificuldades para a obtenção do DNA de boa qualidade, para a interpretação dos resultados, principalmente para avaliar matrizes complexas como as dos queijos (ERCOLINI et al., 2012; ACHILLEOS; BERTHIER, 2013). Contudo, a classe representativa das BAL presentes nos produtos lácteos e suas propriedades tecnológicas pode ser também bem caracterizadas com o uso dos métodos tradicionais de identificação juntamente às técnicas de biotipagem clássicas.

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Capítulo II

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CAPÍTULO II

EVALUATION OF EVOLUTION OF LACTIC MICROBIOTA INVOLVED IN BRAZILIAN BUFFALO MOZZARELLA CHEESE MANUFACTURE

ABSTRACT

The purpose of this study was to evaluate the biodiversity and dynamics of natural LAB involved in Brazilian buffalo Mozzarella cheese during their production and under storage period. The samples were collected during cheese manufacture and storage period from 3 independent trials in a dairy industry located in the southeast region of Brazil. The isolates were characterized by Gram staining, catalase test, by ability of grow at different temperatures (15, 30 and 45 °C), pH (4.5 and 9.6) and under concentrations of NaCl (4.0, 6.5 and 10.0%), and production of CO_2 from glucose. The biodiversity and evolution of LAB was evaluated by 16S rRNA gene sequencing, RAPD-PCR and RFLP-PCR techniques. One hundred fifty-two strains were isolated and LAB showed mainly coccoid and bacilli shape. Most of this strains grow well at 30 °C, are feasible in the presence of 6.5% of NaCl, and in general, the best pH for growing was 9.6. The LAB isolated in this study is typically found in this traditional Italian cheese, except the *Lc. citreum* species. The RAPD-PCR technique allowed evaluating the dynamics of representative LAB in the different steps of Mozzarella production and storage period. Sixty clusters were obtained by RAPD-PCR. All cultures grouped by RAPD-PCR with 85% of similarity (114 cultures) were assessed by RFLP-PCR. Most of the LAB was clustered with 100% of similarity by RFLP-PCR technique. The elucidation of the LAB diversity present in Brazilian buffalo mozzarella cheese enables a better understanding of the evolution of the dominant lactic microbiota during the manufacturing process and storage period.

Keywords: Autochthonous lactic acid bacteria, Raw milk, Biodiversity, RAPD-PCR, RFLP-PCR, 16S rRNA gene sequencing

1. INTRODUCTION

Buffalo Mozzarella cheese is a typical Italian fresh stretched curd cheese (*pasta filata*), that recently has been introduced in the Brazilian market, with good acceptance by its consumers (Silva et al., 2015). Traditionally, this cheese is produced using whole raw milk added or not of natural whey cultures and calf rennet for coagulation step (Losito et al., 2014), followed by a curd-ripening phase (4.0 to 4.5 h at 35 °C to 37 °C), which occurs under whey until optimal pH (4.9 to 5.1). To obtain the "*pasta filata*", the curd is stretched in hot (90 °C to 95 °C) and the elastic product is hand or mechanically molded, which grants its typical round shape (De Candia et al., 2007; Ercolini et al., 2012). It is usually stored into a cold liquid, consisting of water or diluted solution of salts (NaCl and/or $CaCl_2$) (called conditioning brine) and whey (Lucera et al., 2014).

In Brazil, the manufacture of buffalo mozzarella cheese is overall similar to the Italian production; however, it is often manufactured using pasteurized milk added of commercial starter cultures, composed of lactic acid bacteria (LAB) such as *Streptococcus thermophilus* alone, or combined with *Lactobacillus helveticus* and/or *Lactobacillus delbrueckii* subsp. *bulgaricus* (Silva et al., 2015). Only few dairy factories (small facilities) in Brazil follow the typical Italian production process of this cheese using raw milk without addition of commercial culture.

It is known that cheese produced from unpasteurized milk consist of a very diverse and rich microbial ecosystem, in which the LAB composition highly influences the cheeses' quality (Fernández et al., 2010; Franciosi et al., 2009; Marino et al., 2003).

The indigenous lactic acid bacteria present in raw milk have a significant proportion of the microbial communities of most of cheeses (Aydemir et al., 2015) that contribute to acid production during cheese manufacture (De Angelis et al., 2008), as well as, to the particular sensory characteristics, such as flavor and texture (Ercolini et al., 2012; Galia et al., 2009). These characteristics are directly related to various enzymes of the milk but, mainly, to the physiological and biochemical properties of LAB (Franciosi et al., 2009). In order to produce cheese of high quality standards, it is important to characterize in details its natural microbiota.

The study of the biodiversity and the dynamics of natural microbiota LAB during manufacturing and ripening of cheeses represents one of the major interests in cheese microbiology (Randazzo et al., 2009), in which dependent and independent culture techniques

could be considered in determining the specific strains, as well as their possible technological uses (Pogacic et al., 2010; Porcellato et al., 2012; Quigley et al., 2011; Randazzo et al., 2009). Currently, the identification and characterization of microorganisms are based largely on phenotype, physiologic and genetic methods (Ben Belgacem et al., 2009; Ercolini et al., 2012; Lazzi et al., 2009; Randazzo et al., 2009; Silva et al., 2015) and often performed using more than one technique.

Although some studies have been performed to characterize the traditional Italian buffalo Mozzarella cheese (De Angelis et al., 2008; Ercolini et al., 2012; Ercolini et al., 2004; Silva et al., 2015), in Brazil, there is no information available on microbial ecosystem of buffalo Mozzarella cheese. Considering the important role of indigenous lactic microbiota for cheese quality, the purpose of this study was to evaluate the biodiversity and dynamics of natural LAB isolated from Brazilian buffalo Mozzarella cheese during their production and under storage period using a polyphasic approach that involves phenotypic, physiologic and genotypic characterization to identify the LAB at species and strains levels.

2. MATERIAL AND METHODS

2.1. Cheese making

Brazilian buffalo mozzarella cheese was produced using whole raw milk and calf rennet, in a dairy industry located in the southeast region of Brazil. The milk was heated to 35 °C, and the calf rennet was added. Coagulation was allowed to occur for 50 min and then the curd was cut into 1.5-cm^3 pieces and stirred for 20 min. The whey was partially drained, and hot water was added until temperature reached 40 °C, under continuous stirring. After total drained whey, spontaneous fermentation was allowed to occur at room temperature for 24 to 32 h to decline the pH about to 5.0 (optimal pH for stretching). The curd was sliced and hand stretched in water at approximately 80 °C until obtain the elastic curd. After stretching, the mozzarella cheese was mechanically round shaped and packaged in plastic containers with a pasteurized salt solution (NaCl, potassium sorbate solution, and citrate); it was then sealed and stored under refrigeration (4 °C).

2.1.1. Samples collection

Samples were aseptically collected during the steps of manufacture and storage period: raw milk (M), curd (C); streched curd (S), mozzarella cheese after being produced (MC_0), 14 (MC_{14}) and 28 (MC_{28}) days of storage and solution of maintenance after 0 (SM_0), 14 (SM_{14}) and 28 (SM_{18}) days of storage. Samples were collected from 3 independent trials and analyzed in duplicate.

2.1.2. Isolation of LAB strains

Twenty-five grams of each cheese sample were added to 225 mL of sodium citrate solution (2%, w/v) and 10 mL of each liquid sample were added to 90 mL peptone water (0.1%, w/v). Both samples were homogenized for 3 min in a blender (Marconi, Piracicaba, Brazil) at 150 bpm (beats per minute) followed by serial decimal dilutions. About 1 mL from each dilution was pour plated in M17 agar supplemented with 10% of lactose (Himedia, Mumbai, India), and MRS agar 2% acidified with acetic acid at pH 5.4 $MRS_{5.4}$ (Acumedia, Lansing, USA), then incubated for 48 h under aerobic condition at 42 °C, and anaerobic condition provided by Anaerobac (Probac, Sao Paulo, Brazil) at 30 °C, respectively.

After incubation, about 10 different colonies from each plate were randomly selected and analyzed under an optical microscope by Gram staining, and tested for catalase production. Pure isolates (rod and coccoid-shaped bacteria) Gram-positive, catalase-negative, and nonsporogenic were considered LAB, and then preserved with 20% (w/v) glycerol at -80 °C until further analysis (Gatti et al., 2004).

2.1.3. Preliminary characterization of LAB isolates

The cultures were evaluated by ability to grow at different temperatures (15, 30 and 45°C), pH (4.5 and 9.6), as well as the ability to grow in MRS and M17 broth added with different concentrations of NaCl (4.0, 6.5 and 10.0%). The ability to produce CO_2 was determined from glucose in MRS and M17 broth (absence of citrate and supplemented with 5% of glucose) containing inverted Durham tubes (Schillinger and Lucke, 1989).

2.2. Genotypic identification of LAB strains

2.2.1. DNA extraction and quantification

For DNA extraction, the isolated LAB was revitalized overnight in M17 and MRS broth at 42 °C and 30 °C, respectively. Aliquots with 2 mL of each grown culture were pelleted by centrifugation at 16,000 × *g* for 5 min. The genomic DNA was extracted using the Easy-DNA™ extraction kit (Invitrogen, Carlsbad, USA), according to the protocol described by the manufacturer, and quantified at wavelength range 260 to 280 nm in Thermo Nanodrop 2000 (Thermo Scientific, Washington, USA). The resulting purified DNA was stored at -20 °C until use.

2.2.2. Amplification and sequencing of 16S rRNA gene

The 16S rRNA gene was amplified using 2 oligonucleotide primers: fD1 (5'AGAGTTTGATCCTGGCTCAG3'), and rD1 (5'AAGGAGGTGATCCAGCC3'), as described by Weisburg et al. (1991). The PCR conditions consisted of 30 cycles (denaturation step at 94 °C for 1 min, annealing step at 54 °C for 1 min, and elongation step at 72 °C for 1 min), and 1 additional cycle at 72 °C for 7 min as a final chain elongation, according to Silva et al. (2015) and the purification were done according to Sambrook and Russel (2001).

The DNA fragments obtained by PCR were sequenced using the primers fD1 (5'AGAGTTTGATCCTGGCTCAG3') and r16 536 (5'GTATTACCGCGG CTGCTGG3'), according to Muyzer et al. (1993) and to Ercolini et al. (2009), with modifications. All the primers were purchased from Sigma-Aldrich (Munich, Bavaria, Germany). The DNA sequence was determined using the BigDye® Terminator v3.1 kit (Applied Biosystems, Foster, USA) in the 3130 Genetic Analyzer (Applied Biosystems). The nucleotide sequences were aligned and used for the analysis of sequence similarity through Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast) performed using the Gen-Bank database (Natl. Centre of Biotechnology Information - NCBI, http://www.ncbi.nlm.nih.gov/blast).

2.2.3. Genotypic characterization by RAPD-PCR

For the RAPD-PCR analysis, the universal oligonucleotide primer M13 (5'GAGGGTGGCGGTTCT3') was used (Sigma-Aldrich), according to Rossetti and Giraffa (2005) and Silva et al. (2015). Clustering of the resulting patterns was achieved through the unweighed pair group method algorithm (UPGMA), and through the employment of arithmetic averages using the BioNumerics^{TM} software package, version 6.0 (Applied Maths, Sint-Martens-Latem, Belgium). The similarities calculation of the PCR fingerprinting profiles was based on Pearson product-moment correlation coefficient. Based in preliminaries testes (data not shown), strains with a similarity coefficient higher than 85% in the dendrogram were considered belonging to the same biotype.

2.2.4. The tRNA^{Ala}–23S rDNA amplification and PCR purification

Intergenic Spacer Region (ISR) sequences tRNA^{Ala}- 23rDNA of DNA were amplified using tRNA^{Ala} (designed on a conserved sequence of the tDNA^{Ala} of *Oenococcus oeni* (Le Jeune and Lonvaud-Funel, 1997) and 23S/p10 (positions 456–474 of the *E. coli* 23S rRNA gene) primers (Ben Belgacem et al., 2009). The primers were purchased from Sigma-Aldrich. The PCR reactions were performed in a total volume of 50 µL in a GeneAmp® PCR System Thermal Cycler (Applied Biosystems), according to Mancini et al. (2012). For confirmation of DNA amplification, the PCR products were electrophoresed (100 V) in 1.0% (w/v) agarose gel, Tris-borate (EDTA) buffer for 1 h. A DNA ladder of 100 pb (Invitrogen) was used to confirm the fragment size (±600 pb).

Amplified products (1.5 mL) were subsequently purified using sodium acetate (3 mL, 3 M) followed by addition of 100% ethanol (150 µL) and by subsequent precipitation of DNA for 30 min at -80 °C freezer. After precipitation, the DNA was centrifuged at 20,442 *g* for 20 min at 4 °C. The supernatant was discarted, and the precipitate was washed once with 70% ethanol (100 uL). Then, the DNA was centrifuged again at 20,442 *g* for 10 min at 4 °C to obtain the final precipitate. The supernatant was discarted and the DNA (pellet) was suspended in 20 µL of TE buffer (Invitrogen).

2.2.5. RFLP-PCR analysis of $tRNA^{Ala}$–23S rDNA ISR (RFLP-ISR)

The amplified ISR products were digested for 3 h at the optimal temperature for the activity of each restriction enzyme, as indicated by the manufacturer's instructions. For this, three restriction enzymes (*Hind*III, *Hinf*I, and *αTaq*I) (Sigma-Aldrich) were used separately, for each analyzed strain. The digestion reaction was carried out in a final volume of 10 µL, containing: 1 µL TE buffer (10 x), 1 µL of BSA (Bovine Serum Albumin, 100x, Invitrogen), 0.5 µL of enzyme (Sigma Aldrich) and 2.5 µL of water ultra - pure Milli Q (Invitrogen). Restriction products were separated in 2.0% (w/v) agarose gel by electrophoresis at 100 V for 2 h and analyzed using ethidium bromide staining. A DNA ladder (Invitrogen) of 100 bp as a molecular weight marker was used. The images were observed and photographed under UV light using the Kodak Gel Logic 2200 Imaging System (Molecular Imaging Software, WASHINGTON, U.S.A) and saved in TIFF format. The similarity between the generated bands was expressed by Dice coefficient correlation and the UPGMA (unweighted pair-group method using arithmetic averages).

3. RESULTS AND DISCUSSION

3.1. Isolation and preliminary characterization of the lactic microbiota

For isolation of LAB, collected samples were plated and the pure colonies (about 10 different colonies) from each plate were randomly selected and analyzed. From these colonies, those which presented characteristics of LAB (rod, cocci, or coccoid-shape, Gram-positive, catalase-negative, and nonsporogenic bacteria) were purified, and 152 isolates were obtained (Table 1). Most of the strains was isolated from MRS medium at 30 °C and showed coccoid and bacilli shape (data not shown).

All isolated LAB were evaluated for their ability to grow at different temperatures (15, 30 and 45 °C), pH (4.5 and 9.6) and under NaCl concentrations (4.0, 6.5 and 10.0%). The strains were also evaluated for their ability to produce CO_2 by metabolism of glucose (Table 1). The identification of isolates by 16S rRNA sequencing is represented in the Table 2.

Table 1

Characterization of isolated lactic cultures from Brazilian Buffalo Mozzarella cheese samples.

Species	Number of strains	Temperature (°C)									NaCl concentration (%)									pH							CO$_2$
		15			30			45			4			6.5			10			4.5			9.6				
		+	+/-	-	+	+/-	-	+	+/-	-	+	+/-	-	+	+/-	-	+	+/-	-	+	+/-	-	+	+/-	-		+
Enterococcus sp.	61	24	36	1	60	1		61			60	1		38	22	1	19	17	25	18	22	21	57	2	2		
Ent. faecalis	1	1			1			1			1					1			1			1	1				
L. garvieae	1		1		1			1			1					1			1			1	1				
L. lactis	3	1	1	1	3			1	1	1	3					3			3	1		2	3				
Lc. citreum	5	1	3	1	5			2	3		5			1	4			1	4		4	1	5				5
Lc. mesenteroides	38	29	4	5	38			3	22	13	35	3		15	18	5	4	22	12		25	13	32	2	4		38
St. thermophilus	3	2	1		3			3			3					3			3	1		2	2	1			
Lb. casei	20	16	2	2	20			14	5	1	19	1		19	1		13	4	3	19	1		20				
Lb. bulgaricus	10	1	5	4	10			8	2		4	6		3	7		1	5	4	4	6		3	5	2		
Lb. fermentum	9	7	1	1	9			9			9			3	3	3	3	3	3	7	2		9				9
Lb helveticus	1		1		1			1			1			1			1			1					1		

(+) positive and (-) negative growth in 24 h of incubation, (+/-) slight growth in 48 h of incubation.

All *Enterococcus* sp., *Enterocccus faecalis*, *Streptococcus thermophilus*, *Lactobacillus fermentum*, *Lactobacillus bulgaricus* and *Lactobacillus helveticus* strains grew at temperature of 45 °C, while all other cultures showed growth at 30 °C; however, *Lactococcus garvieae* was able to grow at different temperatures as well as the majority of cultures of *Lactobacillus casei*. On the other hand, the strains of *Leuconostoc mesenteroides* grew at 15 and 30 °C, but showed slight growth at 45 °C.

All LAB strains grew at 4% NaCl. *Ent. faecalis*, *L. garvieae, Lactococcus lactis* and *St. thermophilus* showed absence of growth at 6.5% and 10.0% NaCl. Regarding the different pH conditions, cocci and coccoid bacteria showed better growth at pH 9.6, while bacilli grew better at pH 4.5. All strains of *Leuconostoc mesenteroides*, *Leuconostoc citreum, Lb. fermentum* were able to produce CO_2, as expected to heterofermentative LAB (Gaspar et al., 2013).

The characterization of LAB by developing in different conditions (temperature, pH and NaCl content) and by CO_2 production is used to group them into genera or species between cocci and bacilli (Ben Belgacem et al., 2009). However, this classification becomes difficult, as these characteristics may vary and present exceptions, mainly physiological characteristics, for the different species in the same genus. According to Salminen et al. (2004), LAB belonging to the genus *Lactobacillus* are not able to grow at pH 9.6; however, the present results showed that some species of lactobacilli are able to grow in this condition at 24 h of incubation. Additionaly, the *St. thermophilus* cultures in this study grew in 4% NaCl, different from that reported in other studies (Erkus et al., 2013; Salminen et al., 2004).

3.1. Genotypic identification of autochthones lactic microbiota

3.1.1. Strains identification by 16S rRNA gene sequencing

A representative strain from each cluster generated by RAPD-PCR technique was identified by 16S rRNA gene sequencing (Table 2), 62 strains were identified in total. The strains were identified as *Ent. faecalis* (1.61%), *Lc. garvieae* (1.61%), *Lactobacillus helveticus* (1.61%), *Lc. lactis* (4.83%), *St. thermophilus* (4.83%), *Lc. citreum* (8.06%), *Lactobacillus delbrueckii* subsp. *bulgaricus* (8.06%), *Enterococcus sp*. (11.29%), *Lb. fermentum* (11.29%), *Lb. casei* (14.51%) and *Lc. mesenteroides* (32.25%).

Table 2

Identification of representative strains from each cluster generated by RAPD-PCR technique isolated from milk and cheese samples using 16S rRNA gene sequencing.

Strain	Closest relative strain[a]	% Match	Accession number[b]	Fragment size (pb)[c]
SJRP02	*Streptococcus thermophilus*	99	HQ721259.1	446
SJRP04	*Enterococcus* sp.	99	AB680076.1	540
SJRP11	*Enterococcus* sp.	99	AB680076.1	560
SJRP16	*Enterococcus* sp.	100	AB680076.1	568
SJRP30	*Lactobacillus fermentum*	100	HQ293035.1	564
SJRP31	*Leuconostoc citreum*	100	NR074694.1	561
SJRP32	*Lactobacillus fermentum*	100	KC166144.1	559
SJRP40	*Lactobacillus fermentum*	100	HQ29305.1	574
SJRP41	*Lactobacillus fermentum*	99	KC166144.1	572
SJRP43	*Lactobacillus fermentum*	99	KC456368.1	402
SJRP44	*Leuconostoc citreum*	99	NR074694.1	623
SJRP49	*Lb. delbrueckii* subsp. *bulgaricus*	99	NR075019.1	588
SJRP50	*Lb. delbrueckii* subsp. *bulgaricus*	99	NR075019.1	588
SJRP51	*Lb. delbrueckii* subsp. *bulgaricus*	100	JN675227.1	564
SJRP54	*Leuconostoc mesenteroides*	100	AB680227.1	479
SJRP58	*Leuconostoc mesenteroides*	99	HM059008	650
SJRP62	*Leuconostoc mesenteroides*	99	CP000414.1	616
SJRP63	*Leuconostoc mesenteroides*	100	AB680227.1	647
SJRP64	*Leuconostoc mesenteroides*	100	HM059008	571
SJRP66	*Lactobacillus casei*	100	NR075032.1	571
SJRP69	*Enterococcus faecalis*	100	JX307111.1	592
SJRP81	*Lactobacillus fermentum*	100	HQ293035.1	570
SJRP99	*Lactococcus lactis*	99	HM04215.1	477
SJRP101	*Enterococcus sp.*	100	AB680076.1	588
SJRP107	*Streptococcus thermophilus*	100	AY687383.1	599
SJRP109	*Streptococcus thermophilus*	100	NR0748227.1	568
SJRP120	*Enterococcus sp.*	99	AB680076.1	597
SJRP122	*Enterococcus sp.*	99	AB680076.1	540
SJRP125	*Enterococcus sp.*	100	AB680076.1	530
SJRP126	*Lactococcus garvieae*	100	AB598960.1	612
SJRP132	*Leuconostoc mesenteroides*	100	HM218732.1	590
SJRP136	*Lactobacillus casei*	100	NR075032.1	622
SJRP137	*Leuconostoc citreum*	100	HM058680.1	580
SJRP140	*Leuconostoc citreum*	100	HM058680.1	463
SJRP141	*Lactobacillus casei*	99	AB531131.1	498
SJRP144	*Lactobacillus casei*	100	NR075032.1	590
SJRP145	*Lactobacillus casei*	99	NR075032.1	629
SJRP146	*Lactobacillus casei*	99	AB008204.1	632
SJRP148	*Lactobacillus casei*	99	HM05859.1	624

Strain	Closest relative strain[a]	% Match	Accession number[b]	Fragment size (pb)[c]
SJRP149	*Lb. delbrueckii* subsp. *bulgaricus*	100	JN675227.1	500
SJRP150	*Leuconostoc mesenteroides*	100	NR074957.1	570
SJRP154	*Leuconostoc mesenteroides*	100	NR074957.1	575
SJRP156	*Leuconostoc mesenteroides*	100	NR074957.1	539
SJRP160	*Leuconostoc mesenteroides*	99	NR074957.1	588
SJRP161	*Leuconostoc mesenteroides*	100	NR074957.1	597
SJRP163	*Leuconostoc mesenteroides*	99	NR074957.1	630
SJRP164	*Lactobacillus fermentum*	100	KC242235.1	590
SJRP165	*Leuconostoc citreum*	100	AB681815.1	551
SJRP166	*Lactobacillus casei*	100	AB008205.1	648
SJRP167	*Leuconostoc mesenteroides*	100	NR074957.1	619
SJRP169	*Lactobacillus casei*	100	NR075032.1	619
SJRP172	*Leuconostoc mesenteroides*	99	NR074957.1	631
SJRP173	*Leuconostoc mesenteroides*	99	NR074957.1	637
SJRP174	*Leuconostoc mesenteroides*	99	NR074957.1	459
SJRP175	*Leuconostoc mesenteroides*	99	NR074957.1	609
SJRP177	*Lactococcus lactis*	99	HM462395.1	562
SJRP179	*Lactococcus lactis*	99	FJ749558.1	640
SJRP181	*Lb. delbrueckii* subsp. *bulgaricus*	99	JN245656.1	603
SJRP186	*Leuconostoc mesenteroides*	100	NR074957.1	625
SJRP188	*Leuconostoc mesenteroides*	99	NR074957.1	640
SJRP191	*Lactobacillus helveticus*	100	JF728273.1	578

[a]Determined by 16S-rRNA BLAST; [b]GenBank; [c]The fragment length is reported with an approximation of ±1 bp.

The analysis of the 16S rRNA gene allows to identify relatively small differences among species thus enables differentiate BAL species and/or subspecies (Botina et al., 2006). Species belonging to the genera *Lactobacillus*, *Leuconostoc* and *Enterococcus* were reported as dominant microbiota in buffalo Mozzarella cheese by Coppola et al. (1988), Coppola et al. (1990), and isolated by Ercolini et al. (2004), Ercolini et al. (2001), De Candia et al. (2007) and Silva et al. (2015). These LAB were also dominant in whey starter used in the manufacturing of this typical Italian cheese (Coppola et al., 2001; Ercolini et al., 2012; Morea et al., 1998). The presence of *St. thermophilus* and *L. lactis* have also been reported in buffalo Mozzarella cheese (Ercolini et al., 2012). Despite the variations in raw material employed, agroecosystem of the area of production and manufacturing technology, in our study, the Brazilian buffalo mozzarella cheese manufactured with raw milk showed a typical LAB community previously reported in the traditional cheese produced in Italy.

In addition, is interesting to note the presence of *Lc. citreum* isolated from samples of curd (C) and Mozzarella cheese (MC_{14}) during of storage, since there are no reports to date

involving this microorganism in buffalo Mozzarella cheese production; however, *Lc. citreum* has been reported in others fresh cheeses (Cibik et al., 2000), and in other kind of cheeses produced using raw milk (Alegría et al., 2013). According to Alegría et al. (2013), this microorganism is often isolated from cheese produced from raw milk and has shown good behavior in milk although it is absent from the portfolio of most commercial starter culture manufacturers.

Overall, the lactic microbiota present in cheese is complex and beyond, mainly, potentially contribute positively to the control of pathogenic microorganisms (Suskovic et al. 2010) by the production of compounds, like organic acids (lactic, acetic, formic, propionic, and butyric acids), or other compounds, such as carbon dioxide, ethanol, fatty acids, acetoin and diacetyl (Oliveira et al., 2015), that are also responsible for the sensory characteristics of flavor and texture (Pogacic et al., 2010).

3.1.2. Clustering of LAB strains by RAPD-PCR and RFLP-PCR

With the aim of clustering LAB strains with genotypic relatedness, all strains identified by 16S rRNA gene sequencing were fingerprinted by RAPD-PCR. Based on the polymorphisms of bands patterns at a similarity level of 85%, the dendrogram revealed 60 clusters, showing a high biodiversity among strains and species present in a complex system like Mozzarella cheese. The number of strains for each cluster is indicated in parentheses in the Figure 1.

The diversity and the evolution of species collected in the same dairy in three independent trials (different periods) is shown in Table 3. Strains of *Lc. mesenteroides*, *Enterococcus sp.* and *Lb. casei* were present in most of samples and collections. The strains of *Lb. casei* were isolated from the most samples collected.

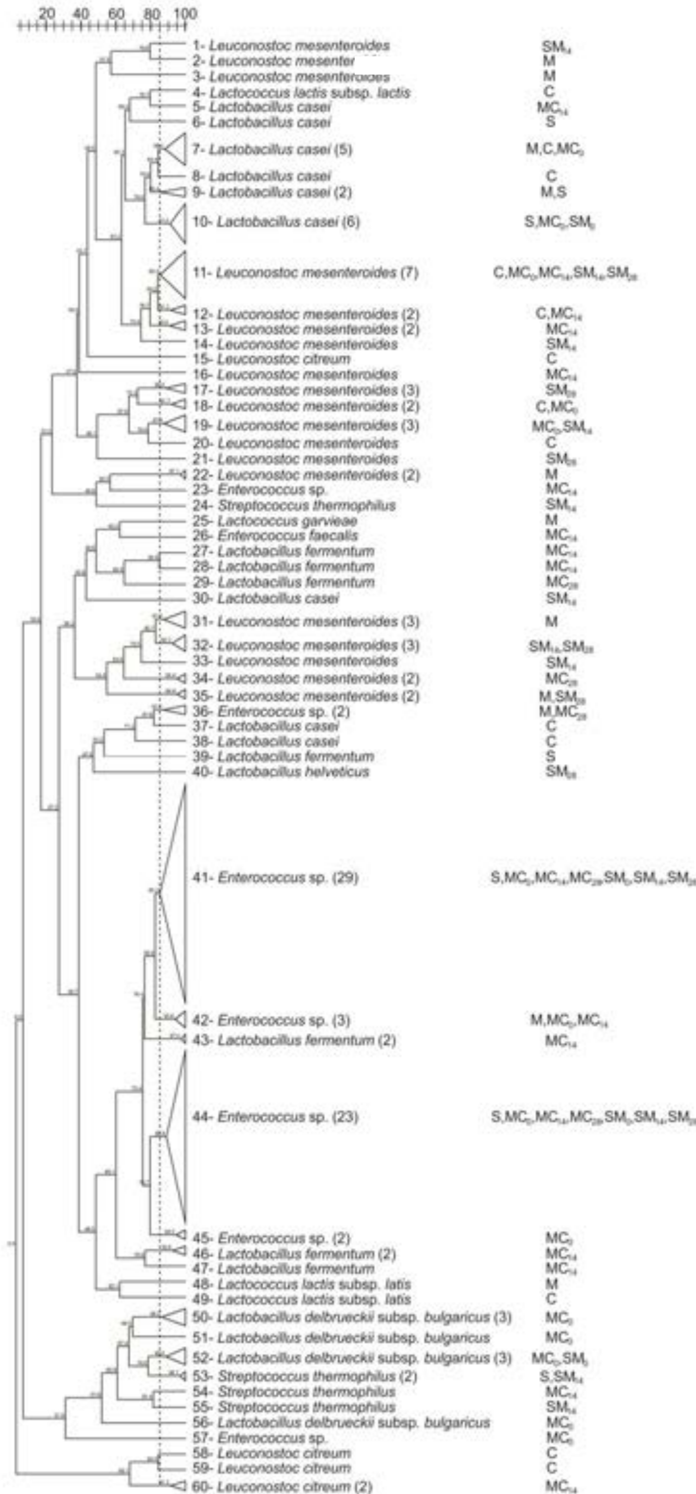

Fig. 1 Dendrogram obtained from M13 RAPD-PCR fingerprints of 152 LAB strains isolated from Brazilian buffalo Mozzarella cheese. Patterns were grouped with the unweighted pair group algorithm with arithmetic averages (UPGMA) based on the Pearson product-moment correlation coefficient. Strains with a similarity coefficient higher than 85% in the dendrogram were considered belonging to the same biotype. Samples: M - raw milk, C - curd, S - stretched curd, MC_0, MC_{14}, MC_{28} - Mozzarella cheese after being produced, and at 14 and 28 days of storage, respectively, SM_0, SM_{14}, SM_{28} - solution of maintenance after being produced, and at 14 and 28 days of storage, respectively.

Table 3

Strains isolated from samples collected in the same dairy in three different periods (I, II and III).

Samples	Species	Total	Collection		
			I	II	III
Raw milk (M)	*Lc. mesenteroides*	9	3	2	4
	Enterococcus sp.	2	1	1	
	L. lactis	1	1		
	L. garvieae	1		1	
	Lb. casei	2	1	1	
Curd (C)	*Lc. mesenteroides*	5	2	1	2
	Lc. citreum	3	2		1
	L. lactis	2		2	
	Lb. casei	5	1	1	3
Stretched curd (S)	*Enterococcus* sp.	6	4		2
	Lb. casei	5	2	3	
	Lb. fermentum	1			1
	Lb. bulgaricus	1		1	
Mozzarella cheese after being produced (Mc_0)	*Lc. mesenteroides*	1	1		
	Enterococcus sp.	15	5	3	7
	Lb. casei	3	1	1	1
	Lb. bulgaricus	5	3	1	1
Solution of maintenance after being produced (S_0)	*Enterococcus* sp.	5	3	1	1
	Lb. casei	2	2		
	Lb. bulgaricus	2		2	
Mozzarella cheese at 14 day after production (MC_{14})	*Lc. mesenteroides*	5		5	
	Lc. citreum	2		2	
	Enterococcus sp.	8	4	2	2
	Ent. faecalis	1	1		
	Lb. casei	1	1		
	Lb. fermentum	7	3		4
	S. thermophilus	1			1
Solution of maintenance at 14 day after production (S_{14})	*Lc. mesenteroides*	10	2	8	
	Enterococcus sp.	10	3	7	
	Lb. casei	1	1		
	Lb. bulgaricus	1	1		
	S. thermophilus	2			2
Mozzarella cheese at 28 day after production (MC_{28})	*Lc. mesenteroides*	2	1	1	
	Enterococcus sp.	6	1	4	1
	Lb. fermentum	2	1		
Solution of maintenance at 28 day after production (S_{28})	*Lc. mesenteroides*	6	6		
	Enterococcus sp.	9	3	5	1
	Lb. bulgaricus	1			1
	Lb. helveticus	1			1

The evolution of LAB strains in the raw milk, during the manufacturing steps, in the Mozzarella cheese and in the solution of maintenance is also illustrated in Figure 1. Strains belonging to the same biotype (clusters 7, 9, 12, 18, 19, 32, 35, 36, 41, 42, 44, 52 and 53) were isolated from different samples during cheese processing. These results also demonstrate the viability of *Lb. casei* and *Enterococccus sp*. (Clusters 7, 37, and 42) isolated from raw milk that remained during the processing steps and in the cheese and/or during the storage period. According to Steele et al. (2013), the lactic cultures associated with the cheeses made with raw milk are considered crucial to generate important sensory characteristics directly

related to cheese quality. *Lc. mesenteroides* was prevalent (except in sample after curd stretching) along the Mozzarella cheese making and during the storage time. The absence of *Lc. mesenteroides* in samples collected after the curd stretching may be due to their intolerance to high temperatures (Hemme and Foucaud-Scheunemann, 2004), as observed previously in the preliminary characterization (Table 1).

Only few strains of *Enterococcus sp*. were isolated from raw milk; however, there was a prevalence of *Enterococcus sp*. after the curd stretching step. Considering the ability of the species of *Enterococcus sp*. to produce biofilms (Didienne et al., 2012; Lortal et al., 2009) and to tolerate high temperatures (Gomes et al., 2008), probably this microorganism was present as a contaminant in the equipment used for curd stretching and, after this stage, became part of the cheese microbiota. On the other hand, the diversity of LAB (*Lactococcus*, *Leuconostoc* and mesophilic *Lactobacillus,* like *Lb. casei*) in microbial biofilms can prevent the development of pathogens in the dairy industries by the production of antimicrobial compounds, such as organic acids, diacetyl, CO_2, bacteriocins and peroxides (Didienne et al., 2012; Suskovic et al., 2010).

Lb. casei strains were found by Morea et al. (1998) during the cheese making process of buffalo Mozzarella. In our study, strains of *Lb. casei* attended over Mozzarella cheese processing; however, they were not viable on the 28-day of storage.

In this study, strains of *L. lactis* were isolated only from raw milk and curd samples, and according to Ercolini et al. (2012), this LAB has been reported as dominant in buffalo raw milk used in the production of Mozzarella cheese.

The diversity and dynamic of LAB strains were revealed by RAPD-PCR technique, which has been successfully useful to identify the microbiota present in complex environments such as cheeses (Lazzi et al., 2009; Mancini et al., 2012; Randazzo et al., 2009). Moreover, the universal M13 primer (used in our study) has been extensively used for biotyping of LAB in dairy products by RAPD-PCR by several authors (Albano et al., 2009; Aponte et al., 2013; De Dea Lindner et al., 2008; Freitas et al., 2015; Gatti et al., 2004; Mancini et al., 2012; Ricciardi et al., 2015; Rossetti and Giraffa, 2005), and it was successfully used in this study to show the diversity between the species and to group the strains belonging to the same biotype. Recent study (Silva et al., 2015) also showed an interesting correlation between the results of RAPD-PCR-M13 and VITEK 2 system for the identification of *Enterococci* strains, hardly identified by 16S rRNA sequencing, indicating the efficiency of physiological and genotypic technique combination for LAB identification.

In addition, according to some authors (Aquilanti et al., 2007; Monfredini et al., 2012; Randazzo et al., 2009; Rossetti et al., 2008), RAPD-PCR is a fast, discriminatory and the most used method for characterizing the diversity of LAB present in dairy products. Therefore, such as the technique of RAPD-PCR, the analysis of intergenic region (ISR) 16S-23S rDNA by RFLP-PCR has been widely used for the identification of LAB in foods. In order to better characterize genotypically the isolates, all strains clustered by RAPD-PCR were also analyzed by RFLP-PCR, 114 cultures in total. For this, the digestion of the region $tRNA^{Ala}$-23 rDNA amplified was carried out using the enzymes *Hind*III, *Hinf*I and α*Taq*I and the similarity between amplicons generated for each enzyme were analyzed using BioNumerics software.

Firstly, the RFLP-PCR results were presented separately in dendograms (data not shown); however, the enzymes *Hind*III and *Hinf*I used individually were not efficient for the differentiation between species of *Lb. fermentum* and *Enterococcus sp*., *Lc. mesenteroides* and *Lb. casei*, respectively. On the other hand, *αTaq*I enzyme, clearly separated the cultures by species. Similar results also were showed by Ben Belgacem et al. (2009).

To further evaluate the results, a single dendrogram was created with the combination of three different RFLP-PCR profiles using the alternative of "composite data" of BioNumerics software. Thus, unlike the individual analysis for enzymes *Hind*III and *Hinf*I, most of LAB was clustered considering 100% of similarity among strains, and they were clearly separated by species and 18 genotype groups presented 100% similarity, whereas 24 strains were not grouped (Figure 2) and some clusters were regrouped when compared with those obtained by RAPD-PCR. The similar comparison between RAPD and RFLP analysis was carried out by Mancini et al. (2012); however they observed low percentage of similarity in clustering the isolates from Grana Padano cheese belonging to the same species.

Additionally, the RFLP-PCR technique is considered more discriminatory than the RAPD-PCR to identify the different LAB species, as well as, more reproductive between laboratories (Mancini et al., 2012). In our study, considering similarity coefficient of 100% (instead of 85%) for clustering strains belonging to the same biotype of LAB in RAPD-PCR, it is obtained 42 clusters (18 clusters with 100% of similarity and 24 strains no grouped), while considering 85% similarity of band profile, 25 different clusters were obtained (13 clusters with similarity ≥ 85%, 6 clusters with 100% of similarity and 6 strains no grouped).

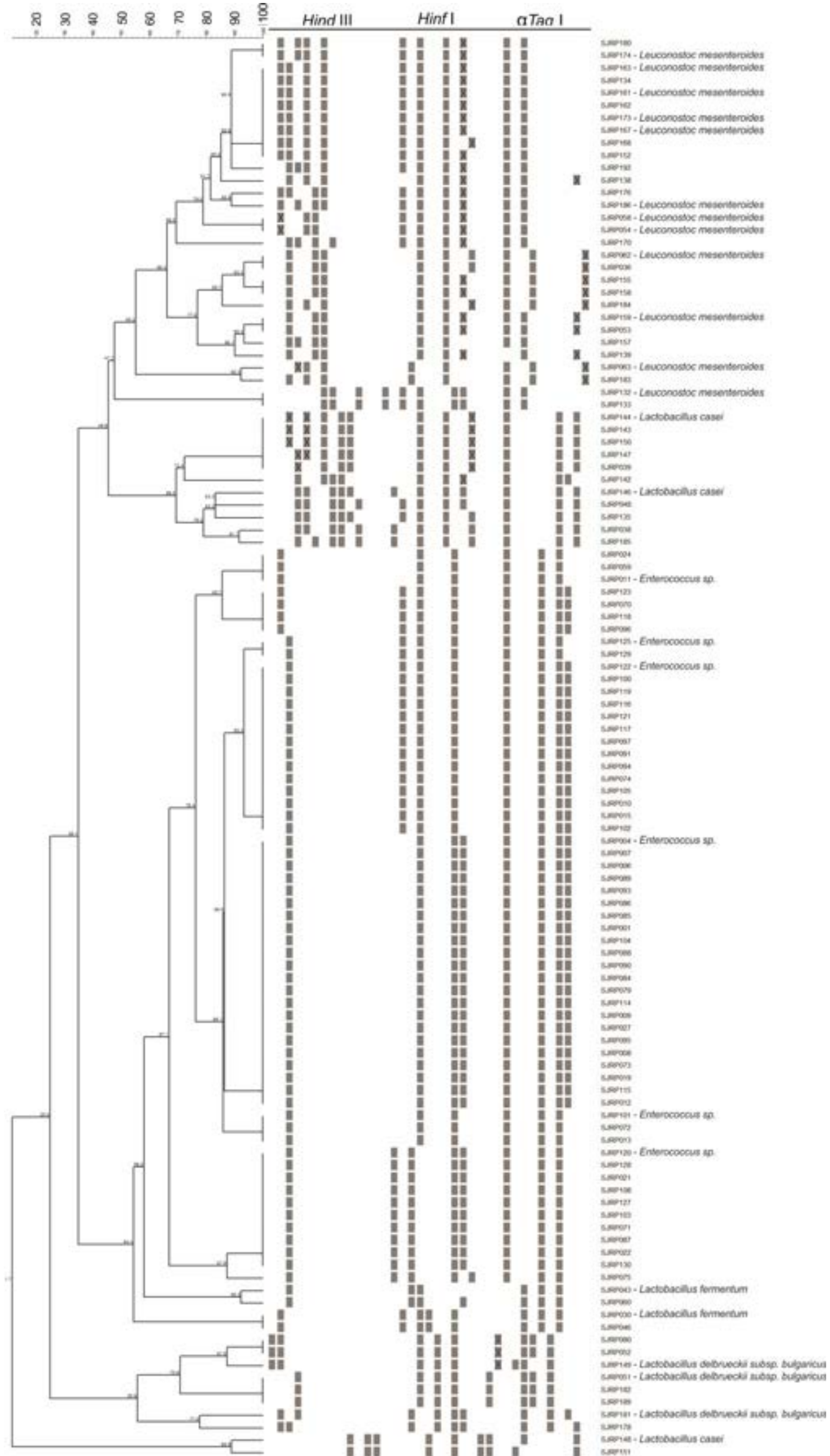

Fig. 2 Dendogram obtained from RFLP-PCR technique from the digestion of tRNA^{Ala}- 23 rDNA region by the combination of enzymes *Hind*III, *Hinf*I e α*Taq*I. Bands with (x) represent uncertain bands that were not considered. Patterns were grouped with the unweighted pair group algorithm with arithmetic averages (UPGMA) based on the Pearson product-moment correlation coefficient.

The RFLP-PCR technique showed coherent clustering compared to those obtained by the RAPD-PCR, since most of the lactic cultures that presented similarity $\geq$ 85% in the same cluster from RAPD-PCR presented 100% similarity by the RFLP-PCR.

In summary, the elucidation of the LAB diversity at species and strains levels present in Brazilian buffalo Mozzarella cheese enables a better understanding of the evolution of the dominant lactic microbiota during the manufacturing process and storage period.

Further studies including the identification of all strains no grouped by RFLP-PCR could explain in more details the diversity of LAB in Mozzarella cheese. Additionally, the isolated strains could be technologically characterized and selected for potential application in cheese production aiming increase the cheese quality.

4. CONCLUSIONS

The isolated lactic cultures were identified as *Ent. faecalis*, *Lc. garvieae*, *Lb. helveticus*, *Lc. lactis*, *St. thermophilus*, *Lc. citreum*, *Lb. bulgaricus*, *Enterococcus sp.*, *Lb. fermentum*, *Lb. casei* and *Lc. mesenteroides*. *Enterococcus* sp., and *Lc. mesenteroides* strains were dominant. Except for *Lc. citreum*, the LAB isolated in this study is typically found in the traditional Italian cheese. The results showed the dynamics of representative LAB in the different steps of Mozzarella production and storage period. The elucidation of the LAB diversity present in Brazilian buffalo Mozzarella cheese enables a better understanding of the evolution of the dominant lactic microbiota during the manufacturing process and storage period using a polyphasic approach that involves phenotypic, physiologic and genotypic characterization to identify the LAB at species (except for *Enterococcus*) and strains levels.

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Capítulo III

Este trabalho será submetido ao *International Journal of Food Microbiology*

CAPÍTULO III

CHARACTERIZATION OF INDIGENOUS LACTIC ACID BACTERIA BASED ON THEIR PRODUCTION OF ORGANIC ACIDS AND ACETOIN IN SKIM MILK

ABSTRACT

Lactic acid bacteria play an important role in the development of the sensory characteristics and biopreservation of dairy products. The present study focus on characterization of sixty-seven indigenous LAB (isolated previously) by their ability to utilize citrate, proteolytic activity, ability to reduce the milk pH and to produce organic acids, acetoin and diacetyl, using High Performance Liquid Chromatography (HPLC), followed by the Principal Component Analysis (PCA). The ability to utilize citrate in differential medium was observed in all tested *Leuconostoc mesenteroides*, *Leuconostoc citreum*, *Lactococcus lactis* and *Lactobacillus fermentum* and in some strains of *Lactobacillus casei*. Most of the strains showed proteolytic activity, were able to reduce the pH to ≤ 5.0 and to produce high concentration of organic compounds. Multivariate principal component analysis of organic acids and acetoin characterized *Lc. mesenteroides* by their ability to produce organic acids (acetic, lactic, formic and pyruvic) and acetoin in skim milk at 30 °C. This analysis differentiated *Enterococcus* sp. strains by their ability to produce acetic, formic and pyruvic acids from *Lb. casei* strains that were characterized by production of lactic acid in skim milk at 37 °C. In skim milk fermented at 42 °C, *Lactobacillus helveticus* and some *Lactobacillus delbrueckii* subsp. *bulgaricus* strains were characterized by their ability to produce acetoin, while the production of lactic acid was related to all *Lb. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* strains and the production of acetic, formic and pyruvic acids was a feature of *Lb. fermentum*. This study reveals the potential of novel indigenous LAB strains to metabolize citrate, and to produce high concentrations of organic acids and acetoin, that can provide desirable aroma and flavor to fermented products, as well as, can contribute to the safety of the final products.

Keywords: Citrate metabolism, Technological application, Flavoring compounds, Principal component analysis, Biopreservation.

1. INTRODUCTION

Lactic acid bacteria (LAB) constitute a microbial group naturally present in the autochthonous microbiota of animal origin foods and are known through ages for their wide applications in food, pharmaceutical, and chemical industries. Several of LAB's are involved in numerous food fermentation processes, possess the "Generally Recognized As Safe" (GRAS) status by the United States Food and Drug Administration (FDA), and are associated with diverse commercial products (Gaspar et al., 2013). LAB are well competitive microorganisms due to their metabolic properties and have a long history of application in preparation of various fermented food products (Penna et al., 2015).

According to the ability to metabolize glucose and type of the final metabolites, LAB are divided into two groups: the homofermentative, which possess aldolase enzyme to convert glucose almost quantitatively to lactic acid via glycolysis or Embden–Meyerhof–Parnas pathway, which are represented by *Lactococcus*, *Pediococcus*, *Enterococcus*, *Streptococcus*, and some lactobacilli; and the heterofermentative, which possess phosphoketolase enzyme, ferment glucose to lactic acid, ethanol/acetic acid, and CO_2 via phosphoketolase pathway consisting in *Leuconostoc*, *Oenococcus*, *Weissella*, and some of lactobacilli (Shiby and Mishra, 2013).

In case of fermentation of lactose (principal carbohydrate in milk), LAB have ability to produce different organic acids, volatile and nonvolatile and aromatic compounds that have an important role in the quality of the fermented food products. Different organic acids are present in fermented dairy products as a result of the hydrolysis of milk fat (free fatty acids such as acetic or butyric), by the production or direct addition as acidulants (citric and lactic acids), by biochemical metabolism (citric, orotic, and uric acids), and/or by bacterial growth (lactic, acetic, pyruvic, propionic and formic acids) (Izco et al., 2002).

In addition, LAB are also able to ferment others carbohydrates such as citrate as an alternative carbon source. According to Bandell et al., 1998, the ability to metabolize citric acid is dependent of permease citrate enzyme (CitP) and can be used by LAB as the sole energy source or be co-metabolized. For heterofermentative LAB like *Leuconostoc mesenteroides* and *Lactobacillus fermentum*, citrate is converted into pyruvate, which is further reduced to lactate. In this case, when citrate is present, more energy is generated during sugar degradation (Schmitt et al., 1992). Particularly for *Lactobacillus casei* (facultative heterofermentative *Lactobacillus*), some stains are able to metabolize citrate when

the presence of glucose is limited. Differently for homofermentative LAB, such as *Lactococcus lactis* and *Enterococcus* sp., pyruvate is the common intermediate formed during sugar and citrate metabolism. The subsequent dissipation of pyruvate leads to the production of C4 (acetaldehyde, acetoin, 2,3-butanediol, diacetyl) aroma compounds (Smid and Kleerebezem, 2014). Additionally, metabolism of lactose and citrate by LAB can produce also significant amounts of ethanol, acetic acid, and other specific flavor related molecules in soft cheeses and fermented milk products (Milesi et al., 2010).

Both pathways (homo and heterofermentative) are used by LAB and the fermentative processes are characterized mainly by the accumulation of organic acids and decreases in pH values (Leite et al., 2013). Besides contributing to the sensory characteristics of dairy products, the production of different organic acids is important for production and for biopreservation of the product due lowering of pH. It is essential to underline, that low pH values are important for casein precipitation during the production of fermented milk products and to particular properties during manufacture of soft cheese, like Mozzarella cheese, that permits an easy plasticization and stretching of the curd in hot water (De Angelis et al., 2008). As well, the low pH has bacteriostatic action against pathogenic microorganisms resulting in the contribution of an extended shelf life and enhanced safety to the products (Penna et al., 2015; Ghosh et al., 2015).

Nowadays, many studies related to the production of flavor compounds and organic acids by LAB have been performed by chromatography analysis followed by multivariate analyses of compounds produced, however, in many cases, these researches were performed using synthetic media similar to food matrices (Pogacic et al., 2015; Sgarbi et al., 2013). Nevertheless, the application of LAB directly in food matrices, such as milk, is more interesting because it can result their real potential application. In this context, the present study focus on the characterization of sixty-seven indigenous LAB from Mozzarella cheese by their ability to utilize citrate in differential medium, proteolytic activity by production of extracellular proteases in agar milk, ability to reduce the milk pH and to produce organic acids, acetoin and diacetyl in skim milk.

2. MATERIAL AND METHODS

2.1. LAB strains

Sixty-seven indigenous LAB strains of different biotype from six species (*Leuconostoc mesenteroides*, *Leuconostoc citreum*, *Enterococcus* sp., *Ent. durans*, *Ent. faecium*, *Lactobacillus casei*, *Lactococcus lactis*, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus fermentum and Lactobacillus helveticus*) previously isolated and identified from different production steps of Brazilian buffalo Mozzarella cheese and storage period were used in this study. All strains belonged to the culture collection of Laboratory of Dairy Technology, Food Engineering and Technology Department, São Paulo State University (UNESP), São José do Rio Preto, SP, Brazil and were maintained as stock cultures at −80 °C in De Man, Rogosa and Sharpe (MRS) (Difco Laboratories, Detroit, MI, USA) or M17 broth (Himedia, Mumbai, MH, India) supplemented with 20% glycerol (w/v; Sigma-Aldrich, Munich, Germany).

2.2. Growth evaluation of LAB strains

The cultures were reactivated from frozen (−80 °C) glycerol stocks in a MRS or M17 broth and then streaked on an agar medium and incubated under conditions described in Table 1. For the determination of pH values and organic acids by HPLC (see below), one colony was collected from each agar plates with a sterile loop and was grown in 6 mL of MRS and/or M17 broth at 30 or 37 °C for 18 h (Table 1). This growth procedure was repeated once. Preliminary experiments were carried out to determine the viable counts of each specie (two or more strains representing each genus/species were tested at different temperatures and incubation times), to ensure inoculation with a similar number of viable cells. For each experiment, fresh cells were used.

2.3. Citrate utilization by LAB strains

The citrate utilization by LAB strains was detected using the differential medium proposed by Kempler and McKay (1980). Citrate-positive colonies were described as blue and citrate-negative colonies were white in color after 48 h of incubation at appropriate

temperatures and under aerobic conditions provided by Anaerobac (Probac, São Paulo, Brazil). *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* ATCC 11007 (citrate-positive) strain was used as a positive control. The analysis was carried out in duplicate.

2.4. Proteolytic activity of LAB strains

The proteolytic activity of LAB strains was qualitatively evaluated on skim milk agar plates according to Pailin et al. (2001). The medium contained 50 g/L peptone (Sigma), 30 g/L yeast extract (Sigma), 12 g/L agar, 10% (v/v) (Difco), skim UHT milk (Molico, Nestlé, Araçatuba, Brazil). The strains from agar plate were inoculated by sterile loop (about 10 μL) and incubated at 30/37 °C or at 42 °C for mesophilic and thermophilic, respectively, for 24 h. After incubation time, the presence of clearing zones indicating proteolysis was recorded. The analysis was carried out in duplicate.

2.5. Inoculum preparation and fermentation

The fermentations were performed in flask using reconstituted skim milk (RSM) (Difco) at 10% (w/v) in sterile distilled water. The strains were initially grown with a 2% starting inoculums in 6 mL of M17 or MRS broth at 30 and 37 °C for 18 h (as described previously). To obtain cells for inoculum, each viable culture was centrifuged twice at 5,000 x g for 6 min at 4 °C, harvested and washed with sterile saline solution (2%, w/v). Each strain was inoculated singly (2% v/v), into RSM to obtain an initial concentration about 10^6 CFU/mL. Experiments were performed in duplicates. After inoculation, the flask samples were incubated at 30-37 or 42 °C during 6 and 18 h (Table 1) for the evaluation of pH value and production of organic acid, acetoin and diacetyl.

2.6. Determination of pH values

Immediately after each fermentation time, an aliquot of fermented milk samples was removed and the pH was measured using a pHmeter model PG1800 (Gehaka, São Paulo, Brazil). Control samples (non-inoculated sterile RSM) were also incubated and analyzed under the same conditions. This experiment was carried out in duplicate. Samples were collected and stored at -80 °C until analysis of organic acids production.

2.7. Analysis of organic acids, acetoin and diacetyl

The analysis of organic acids, acetoin and diacetyl production were carried out by HPLC, using the method described by Donkor et al. (2007). For this analysis, 3.0 mL of fermented milk samples were withdrawn at 6 and 18 h of fermentation and then mixed with 80 μL of 15.5 M nitric acid. Subsequently, the samples were diluted with 1.0 mL of the 0.01 M sulfuric acid (mobile phase used in the HPLC analysis). The resulting mixture was centrifuged at 15,000 × *g* for 20 min at 4 °C using an Eppendorf 5415R centrifuge (Eppendorff, Hamburg, Germany) for removal of proteins. The supernatant was filtered through membrane filters of 0.20 μm-pore diameter (Millipore, Billerica, USA), in a HPLC vial. The analyses were carried out in a Perkin Elmer chromatograph flexar model (Perkin Elmer, Waltham, USA), and the separation was achieved using the Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, USA). The column was stabilized for at least 3 h before use utilizing the same solution and under the same conditions as used for separation. The quantification of organic acids was performed from standard curves obtained using solutions of pre-determined concentrations (Donkor et al., 2007; Zeppa et al., 2001). Unidentified peaks were not reported.

2.8. Principal component analysis

Principal component analysis (PCA) was carried out to characterize studied LAB strains regarding the production of organic acids and acetoin, besides pH alteration. Considering the possibility of potential application of studied strains in different food fermentation processes, which will require specific temperature and fermentation time, this analysis was carried out separately by mesophilic (30 °C, 37 °C) and thermophilic (42 °C) LAB, as well as by fermentation time. Therefore, the organic acids, acetoin and pH were fixed in columns (variables) and the strains in lines (cases), and the data were standardized in the columns before analysis. The PCA was performed using the correlation matrix and without factor rotation. The statistical package Statistica 7.0 (StatSoft Inc., Tulsa, USA) was used.

3. RESULTS AND DISCUSSION

The LAB strains used in this study were revitalized in MRS and M17 broth and cultivated in plate in the same condition (medium, temperature and anaerobic/aerobic conditions) as previously isolated. To obtain a standardized exponential cell phase inoculum (10^6 - 10^8 UFC/mL), preliminary experiments were performed at different temperatures and incubation times (data not shown). The best incubation condition for each species in the specific media (agar, broth and milk) is described in Table 1. The faster growth at first 12 h was observed for *Enterococcus* sp., *St. thermophilus* and *Lb. helveticus* cultures, while for the other cultures the optimal growth was observed between 12 h to 18 h. According to Pogacic et al. (2015), a crucial step that influences all subsequent results is the adjustment of optimal concentration of viable bacterial cells. From the individual colony, two subsequent revitalizations in broth at the ideal conditions of growth for each species showed, in general, better results for all cultures (Table 1).

In our study, all strains of *Lc. mesenteroides*, *Lc. citreum, L. lactis* and *Lb. fermentum*, and most of *Lb. casei* (except the *Lb. casei* SJRP66) generated positive-citrate profile, as been expected. It was known that the ability of metabolize citric acid is dependent of permease citrate enzyme (CitP) (Drici et al., 2010) that is present in various genus of LAB, such as *Lactococcus* and *Leuconostoc* (De Leonardis et al., 2013), *Lactobacillus* (Bandell et al., 1998) and *Enterococcus* (Silva et al., 2015).

When citrate is present as the single energy source, uptake occurs via symport of divalent citrate and one proton or via uniport of monovalent citrate (Konings, 2002; Ramos et al., 1994). During the co-metabolism of citrate and a sugar (citrolactic fermentation), CitP catalyzes the exchange (antiport) of divalent anionic citrate and monovalent lactate (Hugenholtz et al., 1993). Citrate uptake is an electrogenic process, which together with the formation of a pH gradient across the cell membrane, results in the formation of a proton motive force and hence generation of metabolic energy (Bandell and Lolkema, 1999). After transport into the cell, citrate is converted into oxaloacetate and acetate by the enzyme citrate lyase (CitL). Oxaloacetate is further converted by oxaloacetate decarboxylase (CitM), yielding pyruvate and carbon dioxide (Hugenholtz et al., 1993).

Table 1.

Indigenous lactic acid bacteria strains isolated from buffalo Mozzarella cheese and conditions of revitalization and growing in agar, broth and milk.

Species	Strains	Agar		Broth		Milk	
		Medium/O_2	T °C/48 h	Medium/O_2	T °C/18 h	O_2	T °C/18 h
Lc. mesenteroides	SJRP54, SJRP58, SJRP62, SJRP63, SJRP64, SJRP132, SJRP153, SJRP154, SJRP156, SJRP159, SJRP160, SJRP161, SJRP163, SJRP172, SJRP173, SJRP174, SJRP175, SJRP186	MRS/AE	30	MRS/AE	30	AE	30
Lc. citreum	SJRP31, SJRP44, SJRP140, SJRP165	MRS/AE	30	MRS/AE	30	AE	30
Enterococcus sp.	SJRP04, SJRP11, SJRP16, SJRP23, SJRP69, SJRP101, SJRP120, SJRP125	MRS/AE	37	MRS/AE	37	AE	37
Ent. durans	SJRP05, SJRP14, SJRP17, SJRP20, SJRP25, SJRP26, SJRP29, SJRP68	MRS/AE	37	MRS/AE	37	AE	37
Ent. faecium	SJRP28	MRS/AE	37	MRS/AE	37	AE	37
Lb. casei	SJRP35, SJRP37, SJRP66, SJRP136, SJRP141, SJRP145, SJRP146, SJRP148, SJRP169	MRS/AN	37	MRS/AE	30	AE	37
Lc. lactis	SJRP99, SJRP177, SJRP179	M17/AE	37	M17/AE	30	AE	37
St. thermophilus	SJRP107, SJRP109	M17/AE	42	M17/AE	37	AE	42
Lb. bulgaricus	SJRP49, SJRP50, SJRP57, SJRP76, SJRP149	MRS/AN	42	MRS/AE	37	AE	42
Lb. fermentum	SJRP30, SJRP32, SJRP41, SJRP42, SJRP43, SJRP81, SJRP164	MRS/AN	42	MRS/AE	37	AE	42
Lb. helveticus	SJRP56, SJRP191	MRS/AN	42	MRS/AE	37	AE	42

MRS - Man Rogosa Sharpe; AE – aerobic atmosphere; AN, anaerobic atmosphere.

The metabolism of citrate by LAB is very important for selection of cultures for food application, because it can results in the production of acetic acid, acetate, formate, ethanol, acetaldehyde, acetoin, 2,3-butanediol, diacetyl and carbon dioxide. Some of these compounds contribute to the development of aroma and flavor in fermented foods like fermented milks and also to the formation of "eyes", which provide the characteristic texture of some cheeses (De Figueroa et al., 2001; Vaningelgem et al., 2006). It is important to mention that citrate metabolism was also shown to be in correlation with the production of aroma compounds from amino acids (Smid and Kleerebezem, 2014). The transamination of amino acids by aminotransferases is an essential step in the formation of aroma compounds by LAB (Smit et al., 2005).

In addition, the studied cultures were also able to produce extracellular protease in Milk agar (except the *Leuconostoc mesenteroides* SJRP160) observed by the formation of hydrolysis halo around the inoculated strains (data not shown). The ability to produce extracellular proteases is a very important characteristic of LAB with potential application in fermented food production (El-Ghaish et al., 2010). In dairy products, these proteases catalyze the hydrolysis of milk proteins, providing the essential amino acids for microbial growth (Fira et al., 2001) and, consequently influencing the texture, flavor and aroma characteristics of fermented products (Shihata and Shah, 2000). Thus, the positive-citrate and proteolytic activity of LAB strains can be considered for choosing the best candidates to produce flavor compounds.

The production of fermented dairy products depends on the correct choice of LAB starter cultures with specific acidification properties. Changes in pH as results of LAB growth have essential role for the organoleptic characteristics and play important role if safety of the final products. Additionally the studied LAB strains were evaluated for their ability to reduce the initial pH of milk (6.2 ±0.3) and for the presence of acetoin, diacetyl and organic acid during 6 and 18 h of fermentation (Table 2). Firstly, the monitored compounds were assessed and quantified in milk without strains (control samples). The following compounds were identified and quantified: citric acid (1494 mg/L), pyruvic acid (37 mg/L), lactic acid (596 mg/L), formic acid (1418 mg/L), acetic acid (193 mg/L) and acetoin (395 mg/L). The used technique was not efficient to quantified others organic acid, like orotic and uric acid, as well as diacetyl.

Overall, most of the strains reduced the milk pH value until 5.0 at 18 h of fermentation. Especially *Enterococcus* sp. SJRP101 and *St. thermophilus* (SJRP107 and

SJRP109 strains) showed an accelerated reduction of the milk pH until 5.0 compared to other tested strains (data not shown). During the fermentation process in skim milk, pH values were progressively decreased, as a consequence of organic acid production.

The compounds that were present in milk (control) were also detected after fermentation period in all samples. Although it is naturally present in milk, the amounts of these compounds produced by LAB cultures were interesting, especially the highest concentration of lactic acid produced by *St. thermophilus* and *Lb. bulgaricus*; as well as, the highest amounts of formic and acetic acid produced by the most *Lb. fermentum* strains. In general, in all fermentations, the production of lactic acid increased in the intervals between 6 and 18 h. However, for the other tested organic acids and acetoin, it was not possible to establish a standard behavior, been a strain specific. In this case, it may be considered the assimilation or conversion of these compounds to others not assessed in this study (Table 2). The organic acids detected in this study are commonly produced by LAB, as well as, were also present in soft cheeses and fermented milk products (Callon et al., 2014; Casarotti et al., 2014).

During the fermentation period (18 h) only 50% of *Lc. mesenteroides* strains reduced the pH value of milk to ≤ 5.0. Low acidification profile is common characteristic for this species. According to Ayad et al. (2004), *Lc. mesenteroides* acidifies milk slowly; however, they are extensively used in the dairy industry for the production of aromatic compounds (Hemme and Foucaud-Scheunemann, 2004) and exopolysaccharides. As expected for this LAB, high or total consumption of citric acid at 18 h of fermentation was detected by HPLC analysis, in addition to high production of acetic acid and acetoin (825 and 422 mg/L, respectively) (Table 2). The lower pH values during fermentation for *Leuconostoc mesenteroides* SJRP64, combined with the highest consumption of acid citric and the highest concentration of lactic, formic and acetic on 6 h of fermentation (4000 mg/L, 2105 mg/L and 760 mg/L, respectively) were observed.

Similar to the tested *Lc. mesenteroides* strains, the investigated *Lc. citreum* strains were characterized as not good acidifying LAB. Moreover, this specie has low level of acid tolerance (Jeong et al., 2013). Only *Leuconostoc citreum* SJRP31 reduced the milk pH to below 5.0 at the end of fermentation (18 h) and did not produce high concentrations of acids. The highest concentration levels of acetic acid and lactic acid were produced by *Leuconostoc citreum* SJRP44 and *Leuconostoc citreum* SJRP140, respectively, which are organic acids

commonly produced by this species (Alfonzo et al., 2013); however, the production of other monitored compounds was not detected (Table 2).

Furthermore, lactic and acetic acids were the main antifungal substances produced by *Lc. citreum* and *Weisella confuse* described by Baek et al. (2012) at concentrations higher than 17.5 mM. These organic acids were shown to be responsible for retarding the growth of *Cladosporium* sp. YS1 and *Penicillium crustosum* YS2 (Crowley et al., 2013).

Table 2

Lactic acid bacteria species and maximum concentrations (mg/L) of organic acid and acetoin.

Species	Citric acid		Pyruvic acid		Lactic acid		Formic acid		Acetic acid		Acetoin	
	6 h	18 h	6 h	18 h	6 h	18 h	6 h	18 h	6 h	18 h	6 h	18 h
Lm	1437	1433	50	58	4000	5935	2105	2057	760	838	412	422
Lci	1284	1140	32	36	3877	5117	1318	1297	590	675	300	288
Ent	1361	1313	58	75	3572	5586	1615	2860	467	700	294	310
Entd	857	551	54	68	1711	3156	1622	1605	425	619	359	295
Entf	720	126	33	51	910	1958	1160	1378	252	549	254	221
Lcl	1432	1163	54	56	2988	5532	1460	991	307	426	419	392
Lbc	1456	1460	51	93	3205	7761	1318	1225	450	676	421	402
St	1233	1375	52	77	4279	6867	2791	2866	101	107	350	366
Lbb	1502	1421	37	67	4325	10286	1221	2615	237	164	400	370
Lbf	648	306	77	99	1031	2078	6038	8863	588	986	312	316
Lbh	1277	1407	41	69	1346	4872	1300	1525	236	166	352	346

Lm - *Leuconostoc mesenteroides;* Lci - *Leuconostoc citreum*; Ent - *Enterococcus* sp.; Entd - *Ent. durans*; Entf - *Ent. faecium;* Lcl - *Lactococcus lactis;* Lbc - *Lactobacillus casei;* St - *Streptococcus thermophilus*; Lbb - *Lactobacillus delb.* subsp.*bulgaricus*; Lbf - *Lactobacillus fermentum*; Lbh - *Lactobacillus helveticus.*

In present study, most of species of *Enterococcus (Enterococcus* sp., *Ent. durans* and *Ent. faecium*) strains (57%) reduced the milk pH ≤ 5 at 18 h of fermentation. Overall, the higher production of lactic acid and the lower pH values was recorded. For instance, the fermentations of skim milk performed by *Enterococcus* sp. SJRP101 and *Enterococcus* sp. SJRP125 strains showed the highest production of lactic acid and the lowest pH values after the fermentation period. In addition, all tested *Enterococcus* sp. strains were able to produce pyruvic and acetic acid. The formic acid was produced only by *Enterococcus* sp. SJRP04, *Enterococcus* sp. SJRP16 and *Enterococcus durans* SJRP120 strains. The production of

acetoin was not observed in none of the tested *Enterococcus* sp, *Ent. durans* and *Ent. faecium* strains. *Enterococcus* sp. is considered a good producer of acids by lactose fermentation and some strains are also able to metabolize citric acid or citrate for the production of various aromatic compounds (Bandell et al., 1998; Sarantinopoulos et al., 2003). Although in this study using the differential medium the use of citrate was not observed, the consumption of acetic acid was detected by HPLC analysis, and significant reduction was observed in most of experiments. The glycolysis and citrate metabolism in some species of *Enterococcus* confers an additional energetic advantage during their growth (Repizo et al., 2013), and result in the formation of acetate, acetaldehyde, diacetyl, acetoin, and 2,3-butanediol from pyruvate. It can hence be important in flavor formation during milk fermentation and further ripening of fermented dairy products (Vaningelgem et al., 2006).

Most of investigated *Lb. casei* strains (90%) reduced the milk pH to ≤ 5 at 18 h of fermentation. These microorganisms are usually used in mixed or pure cultures for production of fermented milks or cheeses which require a slow fermentation processes. The complete, partial or any assimilation of citric acid by these microorganisms were observed (Table 2). The highest concentrations of lactic and acetic acid were produced by *Lb. casei* SJRP145 and *Lb. casei* SJRP169 strains and were associated with the lowest amounts of citric and pyruvic acid and lowest pH values (4.42 and 4.36, respectively). Possibly, these microorganisms produced acetic acid from metabolism of pyruvate and citrate, once *Lb. casei* are able to produce acetic acid from pyruvate under poor nutrient conditions (McSweeney and Sousa 2000). As well as, the consumption of citrate by this species is significantly higher in low pH values (Vaningelgen et al., 2006). On the other hand, the milk fermented by *Lb. casei* SJRP66 strain showed higher pH values (5.52) and higher production of pyruvic acid and acetoin at 18 h of fermentation, probably by metabolism of citrate (data not shown), because this metabolism can result in the production of pyruvate, acetate, and acetoin (Mortera et al., 2013). The production of formic acid from these cultures was not detected.

All investigated *L. lactis* strains reduced the milk pH to ≤ 5.0 during fermentation period. The milk fermented by *L. lactis* SJRP177 strain exhibited the lowest pH (4.38) during fermentation and highest detected concentration of lactic acid. According to Pereira et al. (2010), during fermentation *L. lactis* converts lactose directly into lactic acid, thus making high the rate of lactic acid formation. The partial consumption of citric acid was also observed for *L. lactis* SJRP177 strain while the highest consumption of citric acid was observed by *L. lactis* SJRP99 culture. As well as, the highest concentrations of pyruvic and acetic acids and

acetoin was recorded for *L. lactis* SJRP99. The production of formic acid was not detected in investigated *L. lactis* strains. In milk, *L. lactis* can co-metabolize sugars and citrate as a secondary carbon energetic sources (Zuljan et al., 2014), and leads to CO_2 production and C4 aroma compounds, that can improve the organoleptic characteristics of dairy products (Zuljan et al., 2014; Smid et al., 2014); however, only specific variants of *L. lactis* (i.e., *L. lactis* subsp. *lactis* biovar *diacetylactis*) are capable of citrate utilization. This property is linked to the presence of a plasmid-encoded citrate transporter gene (Rademaker et al., 2007; Smid and Kleerebezem, 2014). In addition, it is also valid the hypothesis of acquisition of the citrate-fermenting capacity by some lactococci, probably resulting from a horizontal transfer of plasmid genes from *Leuconostoc* species (Drici et al., 2010). Acetic acid often is produced by *L. lactis* subsp. *lactis*, and *L. lactis* subsp. *cremoris* (Salminen et al., 2004).

In our study the two tested *St. thermophilus* strains acidified the milk to pH ≤ 5 at 6 h of fermentation (4.8 and 4.7, for SJRP107 and SJRP109, respectively), as well as, showed high production of lactic and formic acids. Small concentrations of pyruvic acid (≤ 77 mg/L) were detected. However, it was not observed production of other organic acids and acetoin in tested *St. thermophilus* strains. In contrast, a reduction of all compounds in comparison to the initial concentration in milk sample (control) was observed. *St. thermophilus* is a LAB often used for production of cheese, yoghurt and other fermented milks, due to its fast metabolism and, when present, is the main responsible for the production of lactic acid (Oliveira et al., 2012). Formic and pyruvic acids are also produced by this species. In fermented products processing like yogurt, *Lb. bulgaricus* and *S. thermophilus* exhibit a symbiotic relationship, in which *St. thermophilus* provides formic and pyruvic acids, important for *Lb. bulgaricus* development. The formic acid produced by *St. thermophilus* is utilized by *Lb. bulgaricus* as a precursor in purine synthesis (Suzuki et al., 1986). Then, *Lb. bulgaricus* leads to *St. thermophilus* growth by peptides or amino acids production (Nishimura et al., 2013).

All cultures of tested *Lb. bulgaricus* reduced the milk pH to ≤ 5 at 18 h of fermentation, with exception of *Lb. bulgaricus* SJRP149. This difference is probably related to smaller counting of viable cells in the inoculum (about 10^6 CFU/mL) than other strains of this species. *Lb. bulgaricus* grows well at acidic pH and often it is responsible for post-acidification in fermented milks. In our study, for most of *Lb. bulgaricus* strains a drastic reduction of the pH in the last 12 hours of fermentation (data not shown) was observed. This species produced the highest concentration of lactic acid (11863 mg/L) at 18 h of

fermentation (Table 2). In contrast, the production of citric acid, formic acid, acetic acid and acetoin was not detected.

None of *Lb. fermentum* strains acidified the milk to pH ≤ 5 at 18 h of fermentation. According to Ayad et al. (2004), this LAB is considered slow in dairy fermentation, probably by production of low amounts of lactic acid, as observed in our study (≤ 2078 mg/L). In contrast, this LAB produced the highest concentration of formic acid (8863 mg/L) and acetic acid (986 mg/L). The production of acetic acid is probably influenced by the acid citric metabolism, also previously reported by others LAB strains, and as well observed for these microorganisms. The production of organic acids was detected in all *Lb. fermentum* strains while acetoin was not observed.

Despite *Lb. helveticus* is not considered generally as good producer of organic acids, in our study this species acidified the milk to pH ≤ 5 at the end of fermentation (18 h). Additionally, the tested *Lb. helveticus* strains were able to produce pyruvic and lactic acid, however, the production of acetoin and other organic acids was not detected. Poor acidifying strains of *Lb. helveticus* can be used as adjunct cultures depending on their other important properties (Sarantinopoulos et al., 2001). The main contribution of *Lb. helveticus* species to dairy products is due their efficient proteolytic system, responsible for the production of enzymes that confer typical characteristics of texture, flavor and aroma to the different products (Broadbent et al., 2011).

To better evidence the relationships among the LAB strains and the organic acids and acetoin, a principal component analysis (PCA) was carried out using the relative amounts of these compounds for all strains reported in Table 1. Firstly, the control sample was included in the analysis (data not shown), but did not show relevance for the results.

For mesophilic LAB cultivated at 30 °C, the first and the second principal components explained, respectively, 44.8% and 33.4% of observed variations (78.2% in total) at 6 h of fermentation (Fig. 1a). The citric, lactic, formic and acetic acids contributed to explain the variance of component 1, while pyruvic, acetoin and pH value explained the variance of component 2. At 18 h of fermentation (Fig. 1b), the first and the second components explained, respectively, 44.0% and 27.4% of observed variations (71.4% in total), and similar results were found (except lactic acid and pH). Additionally, at 6 h of fermentation, pyruvic acid, acetoin and pH value differed *Lc. mesenteroides* SJRP58 from the other LAB, and at 18 h of fermentation, two *Lc. mesenteroides* strains could clearly be distinguished using PCA by the production of acetoin and pyruvic acid (strain SJRP153) and citric and pH value (strain

SJRP163). Furthermore, *Lc. citreum* SJRP31 distinguished from all other LAB in both fermentation time (Fig. 1). This strain consumed totally citric acid and did not produce any compound studied (data not shown).

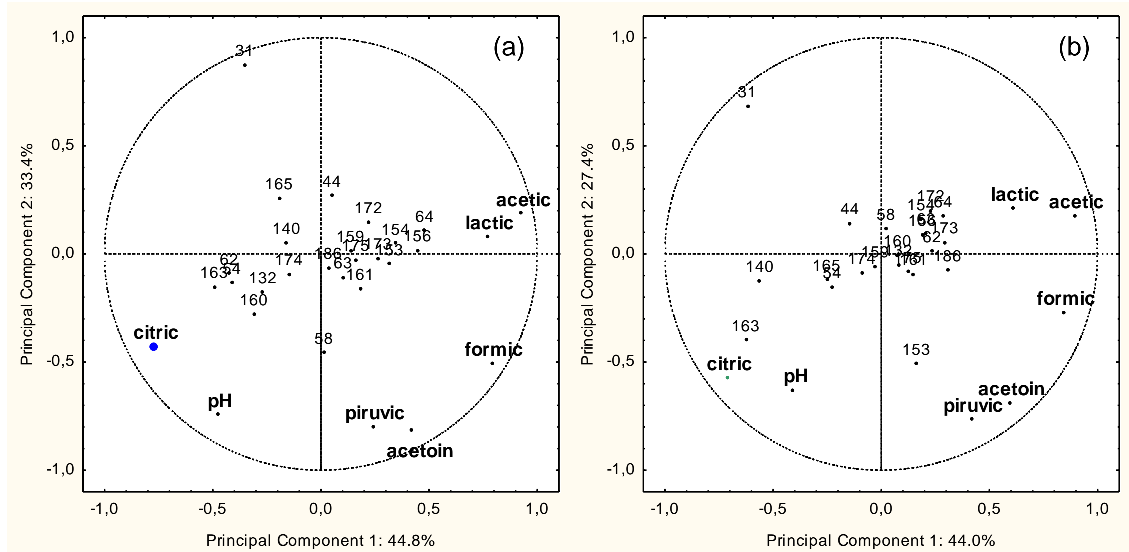

Fig. 1 Results of first and second principal components evidenced by Principal Component Analysis based on the production of organic acid and acetoin, and pH values of milk fermented by mesophilic LAB at 30 °C for 6 h **(b)** and for 18 h **(b)** of fermentation. The codes of the strains are abbreviated; see Table 1 for complete notation of strains.

Leuconostoc sp., especially *Lc. mesenteroides* subsp. *cremoris,* can produce significant amounts of diacetyl and others C4 compounds, like acetoin from citrate present in milk (De Paula et al., 2014). Interestingly, there was high consumption of citric acid by some strains of *Lc. citreum*, like SJRP31; however, without relationship with the production of any compounds analyzed. In this case, probably there was the production of other compounds that were not studied.

For the mesophilic LAB cultivated at 37 °C (*Enterococcus* sp., *Ent. durans, Ent. faecium, Lb. casei* and *L. lactis*), at 6 h of fermentation, the PCA showed that the first and the second principal components described, respectively, 41.1% and 24.3% of the variability (65.4% in total), while at 18 h of fermentation the principal components explained 50.4% (component 1) and 22.2% (component 2), totalizing 72.6% of observed variation (Fig. 2).

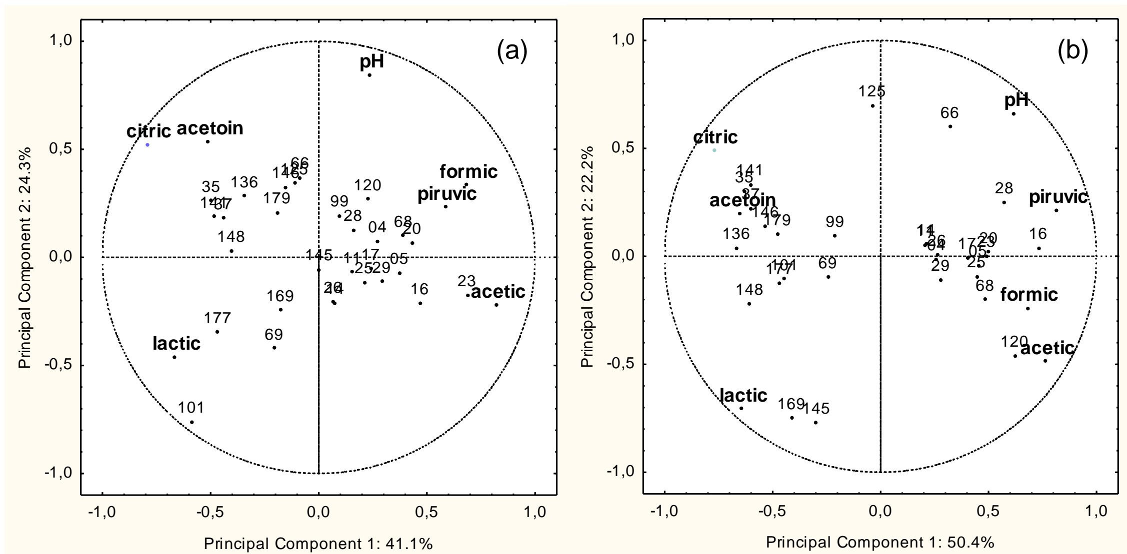

Fig. 2 Results of first and second principal components evidenced by Principal Component Analysis based on the production of organic acid and acetoin, and pH values of milk fermented by mesophilic LAB at 37 °C for 6 h **(a)** and for 18 h **(b)** of fermentation. The codes of the strains are abbreviated; see Table 1 for complete notation of strains.

At 6 h of fermentation (Fig. 2a), citric and acetic acids contributed to explain the variance of component 1, while pH value explained the variance of component 2. *Enterococcus* sp. strains were mainly characterized by the production of acetic acid, specially the strains SJRP16 and SJRP23, probably due to the metabolism of citric acid, while most of strains of *Lb. casei* was distinguished by the presence of citric acid, especially the strain *Lb. casei* SJRP35.

At 18 h of fermentation (Fig. 2b), citric, pyruvic and acetic acids contributed to explain the variance of component 1 that were strongly associated with *Enterococcus* sp. strains, while for component 2, the lactic acid explained the variations. *Lb. casei* SJRP145 and *Lb. casei* SJRP169 differed from other LAB by the lactic acid production, and the amount of this acid was significantly higher (Table 2) compared to the others strains. On the other hand, *Enterococcus* SJRP120 was characterized by the high production of acetic acid.

For the thermophilic LAB, at 6 h of fermentation the PCA showed that the principal components described 80.8% of the variability, being, respectively, 53.3% and 27.5% for the first and second components, while at 18 h of fermentation the principal components explained 63.1% (component 1) and 22.1% (component 2), totalizing 85.2% of observed variation (Fig. 3).

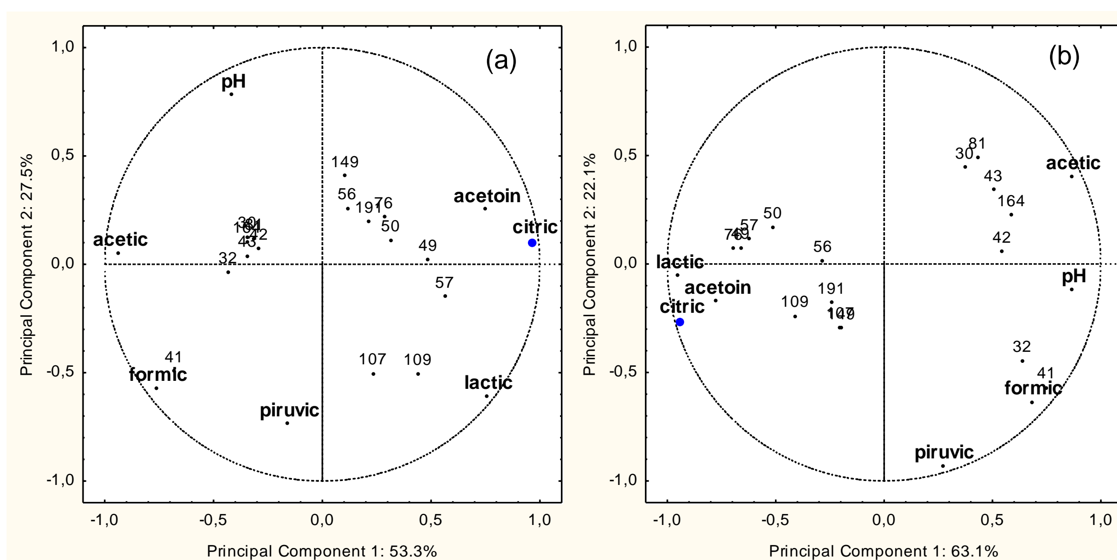

Fig. 3 Results of first and second principal components evidenced by Principal Component Analysis based on the production of organic acid and acetoin, and pH values of milk fermented by thermophilic LAB at 42 °C for 6 h (**a**) and for 18 h (**b**) of fermentation. The codes of the strains are abbreviated; see Table 1 for complete notation of strains.

At 6 h of fermentation (Fig. 3a), citric, lactic, formic and acetic acids and acetoin contributed to explain the variability of component 1, while pyruvic and pH value explained the variance of component 2. *Lb. bulgaricus* and *Lb. helveticus* strains were grouped by acetoin and citric acid. These cultures produced higher amounts of acetoin compared to the other LAB (Table 2), in which citric acid was not produced and nor metabolized. Probably, acetoin was produced by lactose metabolism. *St. thermophilus* SJRP107 and *St. thermophilus* SJRP109 and *Lb. bulgaricus* SJRP57 were distinguished from the other LAB by the high production of lactic acid, as also seen at Table 2. Homofermentative *Lactobacillus* sp. and *St. thermophilus* utilize the glycolytic pathway for the production of energy and converts at least 85% of lactose to lactic acid. In this case, pyruvate is a key intermediate to their metabolism and can be converted into a variety of end products, such as lactic acid, formic acid, acetic acid, acetaldehyde, ethanol, acetoin, diacetyl and butane-2,3-diol (Hickey et al., 1983).

All strains of *Lb. fermentum* were grouped together, except *Lb. fermentum* SJRP41, and separated from the other thermophilic LAB by the production of acetic acid. *Lb. fermentum* SJRP41 was distinguished from all LAB by the production of formic acid. High concentration of formic and acetic acid is produced by this LAB and can contribute to the inhibition of *Listeria* sp. in dairy products (Afzal et al., 2010); however, high amounts of acetic acid result in dairy flavored vinegar, making the product unappealing (Rodrigues et al., 2011).

At 18 h of fermentation (Fig. 3b), citric, lactic and acetic acids and pH value and acetoin explained the variability of component 1, while pyruvic contributed to explain the variance of component 2. *Lb. fermentum* SJRP32 and *Lb. fermentum* SJRP41 strains were grouped together, and separated from the other *Lb. fermentum* strains, which were distinguished by the acetic acid and pH value. These strains produced higher amounts of acetic acid compared to the others LAB, especially the *Lb. fermentum* SJRP30 strain (986 mg/L), probably using the citric acid metabolism. Additionally, despite pyruvic acid could not characterize the *Lb. fermentum* strains, this species produced the highest amounts of pyruvic acid (Table 2).

Previous study also observed the capacity of synthesizing relevant concentration of acetic acid by the *Lb. fermentum* (Ghosh et al., 2015), as well high concentration of pyruvate (Aarnikunnas et al., 2003). Pyruvate is used as an antioxidant, a fat reducing agent, and in the production of polymers and cosmetics.

Lb. bulgaricus strains differed from the other thermophilic LAB and were characterized by lactic and citric acid and acetoin. These strains produced the highest amounts of lactic acid (Table 2).

Finally, the relationship among the species of LAB and the production of the organic acids and acetoin during fermentation at different temperature was better evidenced by PCA. In addition, this relationship helps to select the best species and/or strain to the production of the different products. Furthermore, this study reveals the potential of novel indigenous LAB strains to metabolize citrate, and to produce high concentrations of organic acids and acetoin, that can provide interesting aroma and flavor to fermented products, as well as, can contribute to the safety of the products.

Further studies can also be performed with these strains to estimate the production of other important compounds for better improving the quality of fermented dairy products.

4. CONCLUSION

The ability to metabolize citrate in differential medium was observed to all *Lc. mesenteroides*, *Lc. citreum*, *L. lactis* and *Lb. fermentum* and by some strains of *Lb. casei*. Additionally, except SJRP160 strain, the strains produced extracellular proteases. Most of LAB strains acidified milk to pH $\leq$ 5.0 during 18 h of fermentation and produced organic acids and acetoin; this production is species dependent. Additionally, the relationship between

LAB species and the production of the organic acids and acetoin during fermentations was better evidenced by PCA. This study reveals the potential of novel indigenous LAB strains that can provide interesting aroma and flavor to the fermented product, as well as, can contribute to the safety of the product.

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Capítulo IV

Este trabalho será submetido a *Food Microbiology*

CAPÍTULO IV

SAFETY AND TECHNOLOGICAL APPLICATION OF AUTOCHTHONOUS *STREPTOCOCCUS THERMOPHILUS* CULTURE IN THE BUFFALO MOZZARELLA CHEESE

ABSTRACT

The safety (genes encoding virulence factors and antibiotic resistance) and acidifying activity of autochthonous *Streptococcus thermophilus* cultures were evaluated. The safe culture (free from virulence genes and antibiotic-sensitive) that presented simultaneously good acidification rate was selected and its technological performance was tested in buffalo Mozzarella cheese. Two treatments were conducted: Mozzarella cheeses produced with autochthonous culture (MCSJRP107), and Mozzarella cheese produced with commercial culture (MCSTM5), the control. The chemical composition, texture and proteolysis properties of the Mozzarella cheeses were evaluated. The cultivable lactic acid bacteria (LAB) was evaluated by culture-dependent method (plate counting) and the behavior of *St. thermophilus* cultures (commercial and autochthonous) were evaluated by culture-independent method (RealT-qPCR) during the cheeses manufacture and storage period. The texture, chemical and proteolytic properties were similar for both treatments. Moreover, the MCSJRP107 showed more stability in their texture than MCSTM5 during the storage. The nonstarter LAB count was higher during manufacture than in the storage, and the RealT-qPCR indicated the presence of *St. thermophilus* culture until the end of storage. The autochthonous SJRP107 culture presented high potential for safety application in the production of Mozzarella cheese. Furthermore, considering their relationship with product quality, further studies could be helpful to determine their effect on the sensory characteristics of the cheese.

Keywords: Safety; Lactic acid bacteria; Technological application; Cultivable cells; Quantitative real time PCR.

1. INTRODUCTION

Streptococcus thermophilus is Gram-positive, nonmotile, non-spore-forming, catalase-negative, facultative anaerobic, homofementative lactic acid bacterium, and present low guanine and cytosin (G + C) in the DNA (Goh et al., 2011). It is a thermophilic LAB that is considered as the second most important industrial dairy starter, after *Lactococcus lactis* (Umamaheswari et al., 2014; Douillard et al., 2014). Although being related to other pathogenic streptococci (*e. g. S. pneumoniae* and *S. pyogenes*), *S. thermophilus* is classified as nonpathogenic, possess the "Generally Regarded As Safe" (GRAS) status by the United States Food and Drug Administration (FDA) and have the "Qualified Presumption of Safety" (QPS) status by the European Food Safety Authority (EFSA), due to a long history of safe use in food production (Iyer et al., 2010).

This LAB is traditionally and extensively used as starter cultures in the manufacture of yoghurt, Swiss, French and Italian cheeses like Mozzarella cheese, due to its metabolic traits, such as production of lactic acid, exopolysaccharides (EPS), fermentation of galactose, urease and proteolytic activities, and in some cases, by probiotic and bacteriocinogenic properties (Delorme, 2008; Iyer et al., 2010; Goh et al., 2011; Rossi et al., 2013).

St. thermophilus is the main LAB starter culture used in Mozzarella cheese manufacture, alone or in co-culture with *Lactobacillus helveticus* or *Lactobacillus delbrueckii* subsp. *bulgaricus*. It is always present and in some cases is the most abundant in the natural whey culture (NWC) used in the Mozzarella cheese or in other "pasta filata" cheese manufacture (Coppola et al., 2001; Coppola et al., 2006; De Candia et al., 2007; De Angelis et al., 2008; Ercolini et al., 2012). This microorganism is able to grow or survive at the high temperatures required for Mozzarella cheese production, and it is also mainly responsible for rapid acidification that permits an easy plasticization and stretching of the curd in hot water, and for the formation of the expected flavor and texture properties (De Angelis et al., 2008).

On the other hand, although it presents a good technological profile and being considered safe for use in food, several antibiotic resistance genes have been found in this specie, and probably originated from other LAB (Tosi et al., 2007; Iyer et al., 2010). Essentially, for *St. thermophilus* the presence of acquired antibiotic resistance genes does not pose a significant clinical risk; however, there is the possibility to transfer the antibiotic resistance genes to other species (Tosi et al., 2007). In addition, transferable resistance genes,

especially those located on transposons or plasmid DNA may possess a risk, since they can be transferred to pathogenic bacteria (Aymerich et al., 2006; Devirgiliis et al., 2011).

Furthermore, in general, virulence factors including the presence of virulence and biogenic amine genes must be evaluated to characterize LAB safety (Jeronymo-Ceneviva et al., 2014). Genetic studies of virulence factors include mainly the genes related to surface protein, cell lysis, cellular adhesion and adherence. The biogenic amines are natural toxins of low molecular weight that can occur in fermented foods, and the ingestion of high levels of biogenic amines can be dangerous to human health (Russo et al., 2012; Calzada, 2013). The biogenic amines are often studied and related to histidine decarboxylase, tyrosine decarboxylase and ornithine decarboxylase (Vankerckhoven et al., 2004; Martin-Platero et al., 2009). The decarboxylation of these amino acids results in the formation of the biogenic amines corresponding to histamine, tyramine and diaminobutane (putrescine) (Garai et al., 2007).

To use autochthonous *St. thermophilus* strains in industrial production, it is necessary guarantee their safety. Additionally, it is important to know their physiological, biochemical and genotypic characteristics related with a potential good application. Furthermore, the use of commercially available starter cultures can cause less characteristic cheese flavor. Therefore, the continuous isolation and characterization of new safe starter strains that offer alternative flavor and texture properties has been encouraged. Additionally, the evaluation of viability of the interest cultures can help to explain their relation with cheese quality. For this purpose, plate counting and Polymerase Chain Reaction (PCR) based methods can be well applied (Martinez et al., 2015). Traditional culture-dependent methods have been used for a long time to assess viable and cultivable microbial populations in foods; however, they display an important constraint, since many microorganisms are non-cultivable in synthetic media. More recently, culture-independent molecular methods have been developed to out compete this disadvantage and also speed up the analysis, including real-time quantitative PCR (RealT-qPCR), which is currently considered the most used method for direct quantification of microorganisms (Juste et al., 2008; Barbau-Piednoir et al., 2013).

The RealT-qPCR technique is based on the quantification of a target amplicon marked either with a specific fluorescent probe or a fluorescent DNA-intercalating dye such as SYBR Green (Hanna et al., 2005). SYBR® Green dye binds to all double-stranded DNA, including non-target sequences; thus, for accurate results, SYBR Green assays require a specific amplification that should be confirmed by melting curve analysis. In this case, the conserved

pheS gene has been amplified to identify and quantify the thermophilic LAB, such as *St. thermophilus* (Bottari et al., 2013).

In this context, some studies had been carried out to select interesting starter culture for Mozzarella cheese manufacture; however, few studies are concerned with the safety of the starter culture. The main objective of this study was to evaluate the safety and the acidifying activity of four autochthonous strains of *St. thermophilus* and select a starter culture for testing their technological performances (physicochemical characterization, proteolysis properties, texture, and viability) in buffalo Mozzarella cheese.

2. MATERIAL AND METHODS

2.1. Selection of the culture for cheese making

Four autochthonous *St. thermophilus* strains (SJRP02, SJRP03, SJRP107 and SJRP109) isolated from Brazilian buffalo Mozzarella cheese, previously physiological characterized (data not shown), were technological evaluated for cheese production. The commercial starter culture of *St. thermophilus* (STM5, Chr. Hansen, Denmark) was used to produce the control cheese.

All autochthonous strains belong to the Laboratory of Dairy Technology, from Food Engineering and Technology Department, São Paulo State University, São José do Rio Preto, SP, Brazil and were maintained as stock cultures at -80 °C in M17 broth (Himedia, Mumbai, India) supplemented with 20% glycerol (w/v; Sigma-Aldrich, Munich, Germany). The selection of the culture to Mozzarella cheese manufacture was based on the safety characteristics and acidifying activity (strong requirement for cheese making in industries).

2.2. Safety of the cultures

The safety of the autochthonous *St. thermophilus* cultures was evaluated by investigation the presence of genes encoding virulence factors, vancomycin resistance and biogenic amines often found in lactic acid bacteria, according to Paula et al. (2014). In addition, the sensitivity to antibiotics was evaluated according to Todorov et al. (2011), using the disk diffusion test. For this test, an 18 h-old culture of *St. thermophilus* was inoculated into 20 mL of MRS soft agar (1.0%, w/v; Difco Laboratories, Detroit, USA) to a final

concentration of 10^6 CFU/mL. After solidification, the antibiotic disks (Oxoid, Hampshire, England) were spotted onto the surface of the agar and incubated at 42 °C for 24 h. The inhibitory effect of the antibiotics was expressed in millimeters of the inhibition zones, and cultures with halo ≥ 2 mm were considered sensitive.

2.3. Acidifying activity

The four autochthonous *St. thermophilus* cultures and a commercial *St. thermophilus* culture (STM5, Chr. Hansen, Denmark), often used for Mozzarella cheese manufacture were evaluated. The fermentation was performed using skim powder milk (Difco Laboratories, Detroit, USA) reconstituted to 10% (w/v) in sterile distilled water. The cultures were grown with a 2% starting inoculum in 6 mL of M17 broth (Himedia, Mumbai, India) at 42 °C for 18 h; this growth procedure was repeated once. To obtain cells for inoculum, each viable culture was centrifuged twice at 5,000 x g for 6 min at 4 °C, harvested and washed with sterile saline solution (2%, w/v). Each culture was inoculated singly into reconstituted milk (1%) to obtain an initial concentration of 10^6 CFU/mL. After inoculation, flask samples were transferred to a water bath that was connected to a CINAC system (Cynetique d'acidificacion, Alliance Instruments, Frepillon, France) that allows for the continuous measurement and recording of pH values, as well as the evaluation of the acidification kinetics parameters throughout the run. Batch fermentations were performed at 42 °C up to a pH of 5.0 (fermentation end point).

From the data collected during fermentation, the acidification rate (V_{max}) was calculated as the time variation of the pH (dpH/dt) and expressed as 10^{-3} pH units/min. During the incubation period, the following kinetic parameters were also calculated: t_{max} (h), time at which V_{max} was reached and $t_{pH5.0}$ (h), the time required to reach pH 5.0.

The acidification rate also was calculated as the time necessary to decrease the pH value in 0.4 ($t_{\Delta pH}$ = 0.4 U). The cultures were considered as fast, medium or slow acidifying when a $t_{\Delta pH}$ = 0.4 U was achieved at 3, 3-5 h and > 5 h, respectively (Ayad et al., 2004). This test was conducted in triplicate.

2.4. Application of selected culture in cheese

2.4.1. Selected culture and inoculum preparation

The selected culture for cheese making must be safe (virulence genes free and sensitivity to all antibiotics tested) and present acidifying activity similar to the commercial culture (described above). The inoculum was prepared under the similar conditions used in the test of acidifying activity. The autochthonous culture was reactivated and washed 3 times with sterile saline solution (2%, w/v), harvested by centrifugation (5,000 x *g*, 6 min, 4 °C). The fresh cells were suspended into sterile skin milk (Difco). The commercial culture of *St. thermophilus* STM5 was prepared from lyophilized culture, suspended in pasteurized buffalo milk (20 U/2 L), and then distributed into sterile flasks and stored at -20 °C until use.

Each inoculum with initial concentration of 10^8 CFU/mL was added separately into two fermentation tanks containing 150 L (each) of pasteurized buffalo milk in the proportion of 0.33% and 0.036% (v/v) of *St. thermophilus* SJRP107 and STM5, respectively.

2.4.2. Cheese making

The buffalo mozzarella cheese was produced in a dairy industry located in the southeast region of Brazil, using two separated treatments (MCSTM5 and MCSJRP107), simultaneously, in two independent trials, according to the same conditions. The pasteurized whole buffalo milk was added of 0.025% (v/v) calcium chloride followed by the addition of inoculum (0.036% of STM5 and 0.33% of SJRP107); then, the milk was warmed to 37 °C and the calf rennet was added (0.35%, v/v). Coagulation was allowed to occur for 40 min. The curd was cut into 1.5-cm^3 pieces and stirred for 20 min. The whey was partially drained, and the temperature was warmed to 42 °C until the end of the fermentation process. The fermentation was carried out to decline the pH until 5.0, and the fermentation time was about 4 h for STM5 and 5 h for SJRP107. The final whey was drained and the curd was sliced and hand-stretched in water at 80-90 °C to obtain the elastic product. The temperature of curd reached about 55 °C. After stretching, the matrices was mechanical round shaped and packaged in plastic container immersed in pasteurized salt solutions (0.25% of lactic acid, 2% of calcium chloride and 0.012% of HCl), sealed and stored under refrigeration (at 4 °C).

2.4.2. Collection of cheese samples

Samples were aseptically collected during the steps of manufacture: milk after addition of culture (AC), curd (C); fermented curd ($CpH_{5.0}$) and streched curd (S). The Mozzarella cheese samples were collected after being produced (MC1) and after 5 (MC5), 10 (MC10) and 25 (MC25) days of storage, according to Silva et al. (2015).

2.4.3. Physicochemical characterization of the Mozzarella cheese

After 1 day of production (MC1), the cheese samples were analyzed in triplicate to determine the moisture content, by drying them to a constant weight at 70 °C for 24 h under vacuum (AOAC, 1997); the fat content, using the Gerber-Van Gulik method (Brasil, 2006); the total nitrogen (TN) content, soluble nitrogen in trichloroacetic acid (SN-TCA), and pH 4.6 soluble nitrogen (SN-pH4.6) using the micro-Kjeldahl method (AOAC, 1997) were also performed. The total protein (TP) content was determined from TN content using the conversion factor of 6.38.

After 1, 5, 10 and 25 days of storage, the titratable acidity was assayed and expressed in lactic acid equivalents (AOAC, 1997). It was evaluated through centrifugation of 15 g of samples at 5,700 x *g* for 60 min at 8 ±1 °C (adapted from Harte et al., 2003). The pH values were determined using a pHmeter model PG1800 (Gehaka, São Paulo, Brazil).

2.4.4. Casein fraction determination by urea-polyacrylamide gel electrophoresis (urea-PAGE)

Samples were collected after 1, 5, 10 and 25 days of storage and maintained at -18 °C until the analyses. The cheese samples (20 mg) were incubated at 37 °C for 1 h in Eppendorf tubes with 1 mL of 0.062 M Tris-HCl buffer, pH 6.7, and containing 42 g/100 mL of urea. Thereafter, 5 mL of β-mercaptoethanol were added and the samples were incubated again at 37 °C for 45 min. A drop of bromophenol blue was then added to each sample. Urea-PAGE was performed using a Mini Protean 3 Cell vertical electrophoresis unit (BioRad Laboratories, Hercules, USA). Aliquots of 10 μL were applied to the gel and urea-PAGE was conducted at a constant voltage of 100 V using 0.046 M Tris-glycine, pH 8.3, as the running buffer (Merheb-Dini et al., 2012).

2.4.5. Texture profile analysis (TPA)

After 5, 10 and 25 days of storage, the TPA of the Mozzarella cheese samples was performed using a TA.XT/Plus/50 texture analyzer (Stable Micro Systems Ltd., Godalming, England) to evaluate the parameters of hardness, springeness, cohesiveness and chewiness. A total of 7 cylindrical samples (2.5-cm diameter) were obtained from each treatment. They were prepared a few minutes before analysis and stored under temperature of refrigeration (at 4 °C) in individual plastic containers until analysis. The texture analyzer was equipped with a 4 cm diameter cylindrical stainless probe. The operating conditions were double compression, a crosshead speed of 2 mm/s, and compression of 6.0 mm (adapted from Buriti et al., 2005 and Fritzen-Freire et al., 2010).

2.4.6. Microbiological analyses

Bacterial counts (LAB cultivable cells) of each samples (showed previously) and from each treatment (MCSTM5 and MCSJRP107) were carried out in duplicate from the samples collected. Twenty-five grams of each cheese sample were added to 225 mL of sodium citrate solution (2%, w/v) and 10 mL of each liquid sample were added to 90 mL peptone water (0,1%, w/v). Both samples were homogenized for 3 min in a blender (Marconi, Piracicaba, Brazil) at 150 bpm (beats per minute) followed by serial decimal dilutions. About 1 mL from each dilution was pour plated in M17 agar 2% supplemented with 10% of lactose (Himedia, Mumbai, India) and MRS agar 2% acidified with acetic acid at pH 5.4 - $MRS_{5.4}$ (Acumedia, Michigan, USA) and incubated for 48 h under aerobic and anaerobic conditions at 30 and 42 °C, respectively (Silva et al., 2015). The anaerobic condition was provided by Anaerobac system (Probac, São Paulo, Brazil).

Microbiological safety of Mozzarella cheese samples were examined after 10 days of storage. The presence of total and thermo tolerant coliforms, coagulase positive *Staphylococcus* and *Salmonella* sp. was evaluated according to the American Public Health Association (APHA, 1992).

2.4.7. DNA extraction from the samples and Real time quantitative PCR (RealT-qPCR) analysis

The total DNA was extracted directly from the cheese samples according to the protocol reported by Abriouel et al. (2006), with adaptations. Briefly, 10 mL or 10 g of each sample were added to 90 mL of sodium citrate solution (2%, w/v) and centrifuged for 3 min at 16,000 x *g*. The pellet obtained was re-suspended in TE solution and then 200 µL of TELS buffer (25 mM of Tris-HCL buffer, 10 mM of EDTA, 20 mg/mL of lysozyme and 20% of sucrose [w/v]) (Sigma-Aldrich) and 20 µL of mutanolysin (20 U) (Sigma-Aldrich) were added and treated for 1 h at 37 °C. Then, 25 µL of proteinase K (Sigma-Aldrich) were added and incubated at 70 °C for 30 min. After the treatment, 100 µL of SDS 10% (w/v), 500 µL of phenol pH 8.0 and 100 µL of chlorophorm-isoamyl alcohol (24:1) (Sigma-Aldrich) were added, stirred gentle during 3 min and then centrifuged at 16,000 x *g* for 10 min. The aqueous phase was moved to a new tube, precipitated 3 times with ice-cold isopropanol and stored overnight at -20 °C. The nucleic acids were collected by means of centrifugation at 16,000 x *g* for 10 min, washed briefly in 70% ethanol, centrifuged again and re-suspended in 50 µL of sterile TE added with RNAse (50 µg/L) (Invitrogen, Carlsbad, USA) and incubated by 30 min at 37 °C. The DNA was quantified at wavelength range 260-280 nm in the Thermo Nanodrop 2000 (Thermo Scientific, Washington, USA), diluted up to 20 ηg/µL and stored at -20 °C until use.

The sensibility of the technique and the RealT-qPCR analysis were performed according to Bottari et al. (2013). A master mix was prepared as follows: 1× SYBR® GreenER™ qPCR SuperMix for ABI PRISM® (Invitrogen, Milan, Italy) that contained all the nucleotides, polymerase reaction buffer, and SYBR Green dye, forward StpheSF 5'-GAAGAAATCTTGCTTCGCACTC -3' and reverse StpheSR 5'-'AGGACAGGTTCGAGCATGTGA -3' primers at concentration of 150 nM and nuclease-free water to a total of 20 µL per well. To this, 20 ηg of each DNA from the different samples was added, and the plate was placed in the thermocycler for analysis. Species specific RealT-qPCR was performed using the Applied Biosystems (ABI) Prism 7000 (Applied Biosystems, California, USA) sequence detection system, with fluorescence detection of SYBR Green dye. Amplification consisted of an initial hold at 50 °C for 2 min, 1 cycle of 95 °C for 10 min for denaturation, 40 cycles of amplification (15 s at 95 °C, 60 s at 62 °C). This step was

followed by a dissociation stage (15 s at 95 °C) and melting curves analysis from 60 °C to 90 °C in 20 min and afterwards cooling to room temperature.

The copy number of *phe*S gene for *St. thermophilus* was firstly calculated for the samples in single target real time reactions. Purified DNA of strains of *St. thermophilus* was included in each run to construct standard curves. The sensitivity of the method was estimated measuring the limit of detection (LOD) on the 10, 100, 1000, 5000, 25,000, and 125,000-fold dilution series of the total DNA extracted from the samples.

2.5. Statistical analysis

Test T-student was performed to evaluate the significant differences in the data with regard to chemical composition and analyses of variance (ANOVA) were performed to evaluate the pH value, titratable acidity and texture parameters. Tukey's multiple comparison tests was used to compare the mean values. A probability 5% was used to establish statistical significance. The statistical analyses were conducted using the Statistica 7.0 software (StatSoft Inc., 2004, Tulsa, OK, USA).

3. RESULTS AND DISCUSSION

3.1. Selection of autochthonous *St. thermophilus* culture: safety and acidifying activity

The first criteria for selecting the culture to the Mozzarella cheese production was their safety. Although *St. thermophilus* is classified as a nonpathogenic and GRAS (Burton et al., 2006), the risk of acquiring or transferring genes from and to other bacteria is increased in food system (Kastner et al., 2006; Hummel et al., 2007). In most of the cases, *Enterococcus* and *Streptococcus* have more clinical importance than others LAB. Additionally, the virulence factors enable the bacteria to act as opportunistic pathogens (Ahmadova et al., 2013). In this study, the autochthonous cultures SJRP02, SJRP03 and SJRP107 did not present any of the virulence gene evaluated. In contrast, the strain SJRP109 showed the amplicon to *efaA* and *asa1* genes (Table 1).

Table 1.
Primers sequences used in the investigation of the presence of genes encoding virulence factors, vancomycin resistance and biogenic amine production (Martin-Platero et al., 2009; De Las Rivas et al., 2005; Vankerckhoven et al., 2004).

Targets	Gene	Primers	AT (°C)	Strains			
				SJRP02	SJRP03	SJRP0107	SJRP109
Virulence	*Gel*E	TATGACAATGCTTTTTGGGAT AGATGCACCCGAAATAATATA	47	-	-	-	-
	Hyl	ACAGAAGAGCTGCAGGAAATG GACTGACGTCCAAGTTTCCAA	53	-	-	-	-
	*asa*1	GCACGCTATTACGAACTATGA TAAGAAAGAACATCACCACGA	50	-	-	-	+
	Esp	AGATTTCATCTTTGATTCTTGG AATTGATTCTTTAGCATCTGG	47	-	-	-	-
	*Cyl*A	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT	52	-	-	-	-
	*Efa*A	GCCAATTGGGACAGACCCTC CGCCTTCTGTTCCTTCTTTGGC	57	-	-	-	+
	Ace	GAATTGAGCAAAAGTTCAATCG GTCTGTCTTTTCACTTGTTTC	48	-	-	-	-
Antibiotic resistance	*van* A	TCTGCAATAGAGATAGCCGC GGAGTAGCTATCCCAGCATT	52	-	-	-	-
	van B	GCTCCGCAGCCTGCATGGACA ACGATGCCGCCATCCTCCTGC	60	-	-	-	-
Biogenic amines	*hdc*1	AGATGGTATTGTTTCTTATG AGACCATACACCATAACCTT	46	-	-	-	-
	*hdc*2	AAYTCNTTYGAYTTYGARARGARG ATNGGNGANCCDATCATYTTRTGNCC	50	-	-	-	-
	Tdc	GAYATNATNGGNATNGGNYTNGAYCARGCCRTARTCN GGNATAGCRAARTCNGTRTG	55	-	-	-	-
	Odc	GTNTTYAAYGCNGAYAARACNTAYTTYGT ATNGARTTNAGTTCRCAYTTYTCNGG	54	-	-	-	-

gelE - gelatinase, *hyl* - hyaluronidase, *asa*1 - aggregation substance, *esp* - enterococcal surface protein, *cyl*A - cytolisin, *efa*A - endocarditis antigen, *ace* -adhesion of collagen, *van* A and *van* B - vancomycin resistance, *hdc*1 and *hdc*2 - histidine decarboxylase, *tdc* - tyrosine decarboxylase and *odc* - ornithine decarboxylase. AT - Annealing temperature. (-) Means absence and (+) presence of genes.

These genes are often found in *Enterococcus* sp. (Creti et al., 2004; Perin et al., 2014) and can be easily transferred in milk system. These genes are related to the production of different substances for the adherence and colonization of biotic and abiotic surfaces and also for the invasion of the host immune system (Perin et al., 2014). Additionally, the efa*A* gene is related to cases of endocarditis by some streptococci species (Doyuk et al., 2002).

St. thermophilus SJRP02 and SJRP03 cultures were resistant to oxacillin, unlike the previously related by Karapetkov et al. (2011) for *St. thermophilus* LC201 strain. However, the SJRP107 and SJRP109 cultures did not show resistance to the tested antibiotics (Table 2).

Table 2.

Effect of antibiotics on the growth of autochthonous *St. thermophilus*, presented as diameter of inhibition zones in millimeters.

Antibiotics action (µg/disk)	Active ingredient	Strains			
		SJRP02	SJRP03	SJRP107	SJRP109
Interferes with bacteria cell wall synthesis					
Ampicilin 30	Penicilin	11	11	15	10
Penicilin 10	Penicilin	9	12	16	11
Oxacilin 1	Penicilin	0	0	8	3
Vancomycin 30	Glycopeptide	5	5	6	5
Bacitracin 10	Polypeptide	5	6	10	3
Teicoplanin 30	Glycopeptide	6	10	8	5
Fosfomicin 50	β-lactams	9	9	10	6
Inhibits protein synthesis					
Linezolid 30	Oxazolidinone	11	11	9	9
Gentamicin 10	Aminoglycoside	4	7	5	3
Streptomycin 300	Aminoglycoside	7	8	9	7
Erytromycin 15	Macrolide	10	11	8	9
Tetracycline 30	Tetracycline	10	11	12	5
Chloramphenicol 30	Thiamphenicol	11	9	8	9
Inhibits DNA synthesis					
Nitrofurantoin 300	Nitrofuran	10	9	11	9
Norfloxacin 10	Fluoroquinolone	7	6	5	3
Ciprofloxacin 5	Fluoroquinolone	7	7	7	5

After the safety evaluation, the acidifying activity was performed. This is considered the most important characteristic to the technological application of LAB in Mozzarella cheese production. The acidification profile of pure *St. thermophilus* cultures in milk was characterized by the parameters V_{max}, t_{Vmax}, pH_{Vmax}, $t_{pH5.0}$. The highest V_{max} values were obtained by commercial *St. thermophilus* STM5 (15.23×10^{-3} upH/min) and by SJRP107

culture (13.23 x 10^{-3} upH/min) (Table 3). These cultures were also fast acidifying and reduced the milk pH until to 5.0 more quickly than others autochthonous cultures, as well as they showed very similar acidification profile (data not shown). The SJRP102, SJRP103 and SJRP109 cultures showed lowest V_{max} values and a medium acidification activity. Despite belonging to same species, the *St. thermophilus* cultures showed different acidification profile. It could be due to the different proteolytic system among the strains (Setachaimongkon et al., 2014). Additionally, the starter cultures have to produce lactic acid rapidly and in sufficient concentration to permit an easy plasticization and stretching of the curd in hot water (De Angelis et al., 2008). The rapid acidification (ca. 2-4 h) allows the total time of cheese manufacturing to be shortened, which reduces the total amount of syneresis during cheese making and enables higher moisture content to be achieved in the cheese (Barbano et al., 1994). Moreover, previously this culture was characterized positively for extracellular protease production (data not shown). In milk, these proteases catalyze the hydrolysis of proteins present providing the amino acids essential for their growth (FIRA et al., 2001) and, consequently amending the texture, flavor and aroma of fermented products (Shihata; Shah, 2000).

Based on the safety tests and acidifying activity, the *St. thermophilus* SJRP107 culture showed better results compared to the others autochthonous cultures, and therefore, it was selected for potential technological application in Mozzarella cheese.

3.2. Physicochemical characterization of Mozzarella cheese

The chemical composition of Mozzarella cheese was performed on the first day of production. Statistically, the amount of fat, ash, protein, SN-pH 4.6 and SN-TCA were similar between the two studied treatments (MCSTM5 and MCSJRP107); however, significant difference ($p \leq 0.05$) in moisture content among the treatments was observed (Table 4). Although MCSJRP107 showed higher moisture content, this requirement is accordance to the standards established by Brazilian legislation, which determines moisture ≤ 60 g/100g for Mozzarella cheese (Brasil, 1997). Cheese samples from both treatments were classified as high-moisture traditional cheeses (Jana and Mandal, 2011). In general, the chemical composition observed was in the typical range for buffalo mozzarella cheese (Sameen et al., 2008; Mari et al., 2014).

Table 3.

Kinetic parameters of acidification of *St. thermophilus* during fermentation at 42 °C.

Strains	V_{max} (10^{-3}upH/min)	t_{vmax} (h)	pH_{vmax}	$t_{pH5.0}$ (h)	$t_{\Delta pH=0.4}$	Classification
SJRP02	09.49 (±0.57)	4h33min (±0.00)	5.69 (±0.02)	06h21min (±0.18)	3h25min	Medium
SJRP03	06.19 (±0.03)	3h42min (±0.00)	6.18 (±0.02)	18h23min (±0.17)	3h50min	Medium
SJRP107	13.23 (±0.71)	4h25min (±0.11)	5.51 (±0.02)	05h32min (±0.12)	2h50min	Fast
SJRP109	07.47 (±0.24)	4h02min (±0.08)	6.23 (±0.02)	13h00min (±0.47)	4h25min	Medium
STM5	15.23 (±0.58)	3h21min (±0.41)	5.50 (±0.26)	4h13min (±0.42)	1h58min	Fast

V_{max} - maximum acidification rate; t_{Vmax} - time required to reach V_{max}, pH_{Vmax} – pH in V_{max}; $t_{pH5.0}$ - time required to reach pH 5.0 (end of fermentation), $t_{\Delta pH}$ = 0.4 U - time necessary to decrease the pH value in 0.4.

Table 4.

Chemical composition of buffalo Mozzarella cheeses MCSTM5 and MCSJRP107.

Analysis	MCSTM5	MCSJRP107
Moisture (%)	$56.65^{b}\pm1.01$	$58.78^{a}\pm1.33$
Fat (%)	$22.75^{a}\pm1.75$	$23.50^{a}\pm0.31$
Ash (%)	$1.33^{a}\pm0.21$	$1.40^{a}\pm0.29$
SN pH 4.6 (%)	$0.39^{a}\pm0.12$	$0.36^{a}\pm0.07$
SN TCA (%)	$1.64^{a}\pm0.17$	$1.77^{a} \pm 0.22$
Protein (%)	$17.54^{a}\pm1.32$	$16.26^{a}\pm0.96$

[a, b] Mean values (± standard deviation) in the same row that are indicated by different letters are significantly different ($p \leq 0.05$). n=6. SN pH 4.6 - soluble nitrogen in pH 4.6, SN TCA - soluble nitrogen in TCA (trichloroacetic acid). MCSTM5 - Mozzarella cheese manufactured with STM5 culture, MCSJRP107 - Mozzarella cheese manufactured with SJRP107 culture.

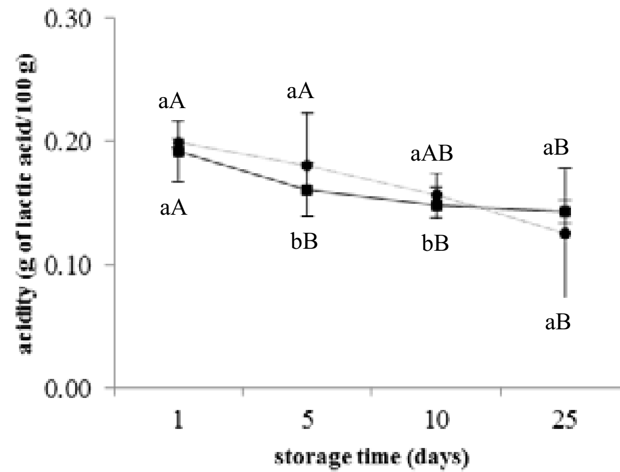
The titratable acidity and pH values were performed after 1, 5, 10 and 25 days of storage (Fig. 1). The acidity of the cheeses varied during analyzed period and was considered statistically equal between the treatments ($p > 0.05$) only on the first (MC1) and on the last day of analysis (MC25); however, a small reduction for both treatments was observed during the storage period. This reduction of cheese acidity may be due the water absorption from the preservative solution. Although the moisture content has not evaluated during storage, it was possible to visualize a larger diameter of the cheeses at the end of the storage period. A similar result was reported by Minervini et al. (2012) for Fior di Latte cheese. Thus, the increase of moisture masks the possible acidification by LAB action during storage, which also decreased during of storage (see below).

In general, pH values were statistically different ($p \geq 0.05$) between cheeses, except for the last evaluated day (25th day); however, this variation may be regarded as normal for cheese manufacturing processes exposed the environment conditions. For both treatments, the pH values were not affected by storage time ($p > 0.05$). Additionally, this result can be interesting because it indicates not occurred post-acidification during refrigerated storage, required by Mozzarella cheese.

The casein fractions did not show progressive hydrolysis during storage period. In all samples were only identified the two main casein fractions. A greater mobility concerning to the α_{S1}-CN and lower mobility on the β-CN fraction (data not shown). Primary and secondary proteolysis affects stretch, melt, flavor, and cook color of non-ripened Mozzarella cheese as well as curd structure during refrigeration. Probably, the hydrolysis did not happened due to

the short storage time at 4 °C and because the Mozzarella cheese is a fresh cheese. Additionally, thermophilic lactobacilli such as *L. delbrueckii* ssp. *bulgaricus* and mesophilic cocci such as *Lactococcus lactis spp.* have a more intense proteolytic activity than *St. thermophilus* (Minervini et al., 2012).

(a)



(b)

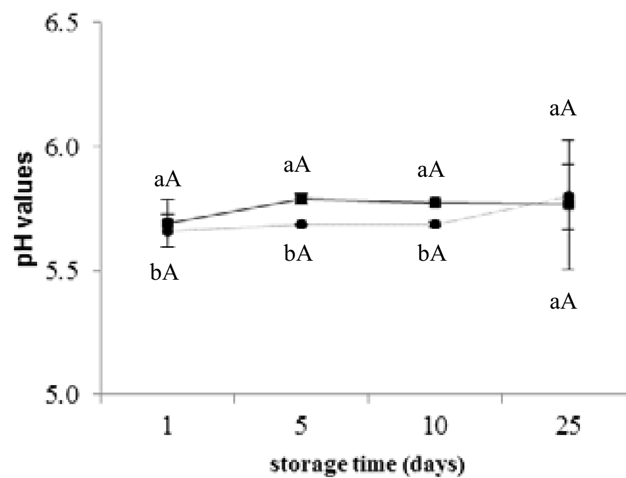


Fig. 1 **(a)** Titratable acidity (g/100 g) and **(b)** pH values of cheeses samples during the storage period (4 °C). —●— MCSTM5 - Mozzarella cheese manufactured with STM5 culture. —■— MCSJRP107 - Mozzarella cheese manufactured with SJRP107 culture. [a, b] Different lower case letters denote significant differences ($P \leq 0.05$) among different sampling periods of the assay for the same treatment. [A, B] For the same storage period, different capital letters denote significant differences ($P \leq 0.05$) among treatments for the same sampling period of the assay. n=6

3.3. Texture profile analysis (TPA)

Texture profile analysis was performed after 5, 10 and 25 days of storage. In general, the texture parameters were very similar between the two treatments on the 5th day of analysis ($p \geq 0.05$); however, during storage time there were some differences (Table 5). During analyzed period, there were no significant changes ($p > 0.05$) for MCSJRP107 cheese, except for cohesiveness on the 5 and 25th days of storage, showing the good potential for industrial application, since the textural characteristics were stable under refrigerated storage. On the other hand, cheese made with STM5 culture showed statistically significant differences ($p \geq 0.05$) for hardness, springeness and chewiness during storage, with decreasing values between the 5th and 25th days of storage. Similar result was observed for Mozzarella (Zisu and Shah, 2007; Dong et al., 2009) and other fresh cheeses (Diamantino et al., 2014). This increase in softness in fresh cheese is largely promoted by the residual coagulant enzyme, which acts during the initial period of storage by hydrolyzing the α_{S1}-casein to produce $\alpha_{S1\text{-}1}$-casein (Fox and McSweeney, 1998). In general, the texture profile observed in this study is similar to the previously reported for Mozzarella cheese by Dong et al. (2009).

Table 5.
Texture profile of buffalo Mozzarella cheeses MCSTM5 and MCSJRP107 during the storage period (4 °C).

Parameters	**Days**	**MCSTM5**	**MCSJRP107**
Hardness (N)	**5**	$13.27^{B,a}\pm1.34$	$12.94^{A,a}\pm1.88$
	10	$16.99^{A,a}\pm1.78$	$12.41^{A,b}\pm 1.15$
	25	$9.29^{C,b}\pm2.33$	$12.59^{A,a}\pm1.24$
Cohesiveness	**5**	$0.70^{A,b}\pm0.04$	$0.73^{A,a}\pm0.05$
	10	$0.66^{A,a}\pm0.04$	$0.68^{B,a}\pm0.06$
	25	$0.70^{A,b}\pm0.07$	$0.73^{A,a}\pm0.06$
Springeness	**5**	$0.72^{AB,a}\pm0.05$	$0.75^{A,a}\pm0.09$
	10	$0.75^{A,a}\pm0.06$	$0.75^{A,a}\pm0.06$
	25	$0.68^{B,b}\pm0.04$	$0.72^{A,a}\pm0.05$
Chewiness (N)	**5**	6.69±0.91	7.07±1.40
	10	8.48±1.59	6.32±1.01
	25	4.35±1.01	6.66±1.11

$^{A, B, C}$ For each parameter, mean values (± standard deviation) in the same column, indicated by different capital letters are significantly different ($p \leq 0.05$). $^{a, b}$ Mean values (± standard deviation) in the same row, indicated by different lower case letters are significantly different ($p \leq 0.05$). n=14. N - Newton. MCSTM5 - Mozzarella cheese manufactured with STM5 culture, MCSJRP107 - Mozzarella cheese manufactured with SJRP107 culture.

Further studies including characterization of antimicrobial compounds and probiotic potential, as well as, the production of organic acids and aromatics compounds could be helpful to determine the effect of *St. thermophilus* SJRP107 culture on the quality and sensory characteristics of the cheese.

3.4. Microbiological analyses

The dynamic of cultivable LAB population was evaluated by plate counts during the two different stages: manufacture and storage period for both cheeses. The population of *Lactobacillus* was grown in $MRS_{5.4}$ agar under anaerobic conditions and the cocci in M17 agar under aerobic conditions, both at 30 °C and 42 °C for 48 h. During processing, the population of thermophilic and mesophilic LAB in the AC (milk after addition of culture) and C (curd) steps for both cheeses remained stable (Fig. 1), except for thermophilic *Lactobacillus* that showed an increase about of 3.0 log cycles.

For the MCSTM5 sample, after fermentation period ($CpH_{5.0}$ - fermented curd step) there was an increase in the cultivable cells numbers, except for the mesophilic *Lactobacillus,* while no changes in counts was observed for the MCSJRP107 cheese. In general, for the MCSTM5 cheese, the S (streched curd) step decreased about 2.0 log cycles in LAB counts. This is a common characteristic due to their sensitivity to heat treatment (Ercolini, 2004). On the other hand, the LAB counts did not decrease in the stretching step in the MCSJRP107 cheese, except for the mesophilic *Lactobacillus*. Despite in general LAB being sensible to heat treatment, some studies showed improvement of LAB viability upon exposure to sub lethal stresses (e.g. heat and cold), which enhances cell resistance during industrial processing (Minervini et al. 2012; Fittipaldi et al., 2012). Furthermore, the heat resistance of some nonstarter LAB (NSLAB) is well known. Thus, LAB found at lower numbers in Mozzarella cheese should be present in the milk and survived during pasteurization process (Minervini et al., 2012).

In the second stage of the analysis, both cheese samples presented lower cultivable LAB cells during storage period (1, 5, 10 and 25 days of storage) than in the samples collected during cheese manufacture. Similar results were found by De Angelis et al. (2008). In general, the *Lactobacillus* counts were lower and drastically reduced in this period, except to MCSTM5 with 5th of storage. This characteristic may be due to the competition between others indigenous cultures and starter cultures added or due to *Lactobacillus* sensibility under

refrigerated storage. In addition, it can also be consider the cell lysis of the LAB by stress undergone under low temperature during the storage period.

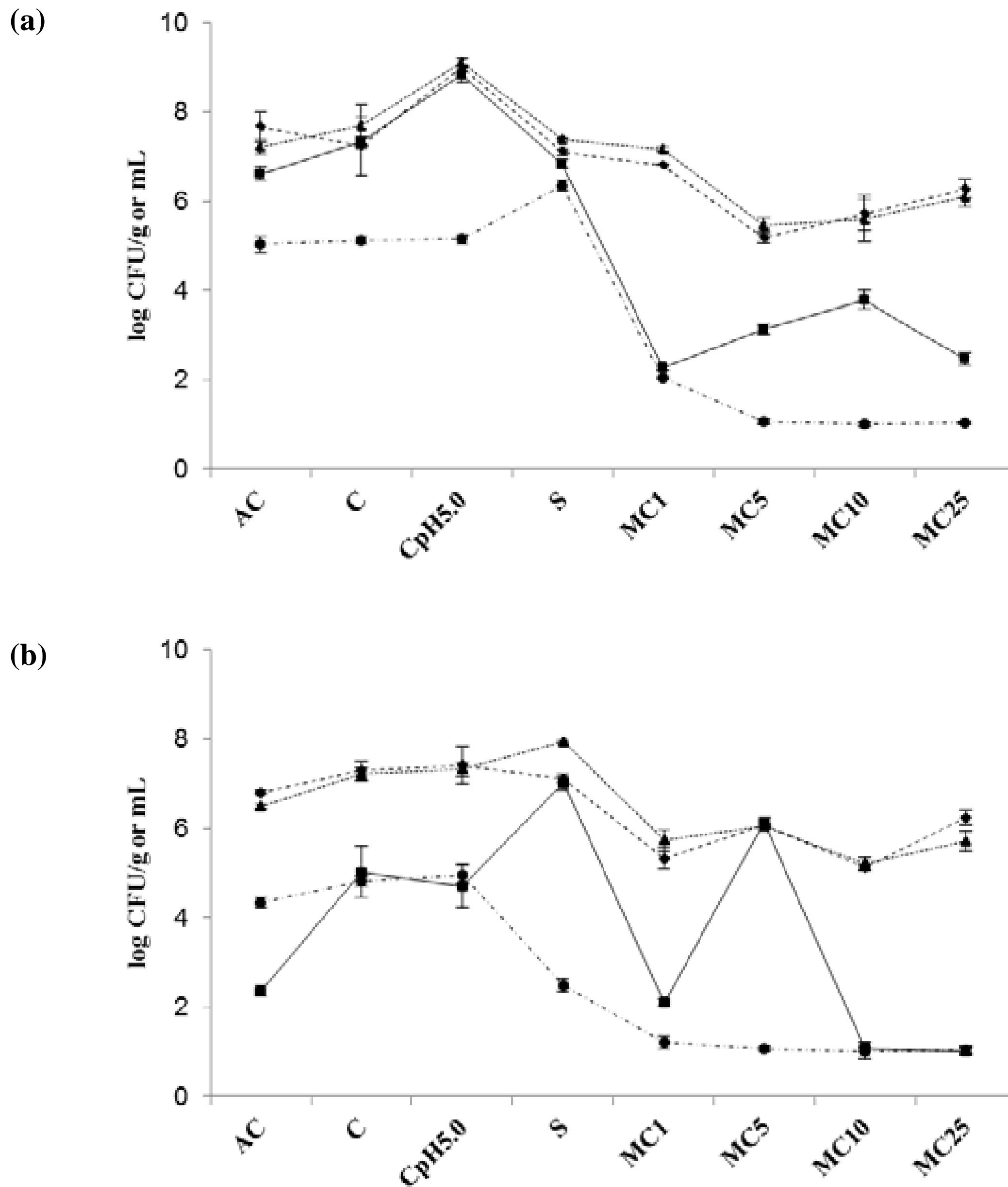


Fig. 2 Evolution of LAB population, expressed as log CFU g or mL, during different steps of buffalo Mozzarella cheese manufacturing and storage period. **(a)** MCSTM5 and **(b)** MCSJRP107. During cheese making the samples were collected from milk after addition of culture (AC), curd (C), fermented curd ($CpH_{5.0}$) and stretched curd (S). The Mozzarella cheese samples were collected after being produced (MC1) and after 5 (MC5), 10 (MC10) and 25 (MC25) days of storage. M17 30 °C; M17 42 °C; IRS 30 °C; RS 42 °C

The microbiological safety of Mozzarella cheese samples was examined according to the requirements established by Brazilian standards (Brasil, 2003) after 10 days of storage.

Both treatments did not show *Salmonella* sp. (absence in 25 g) and neither total nor thermo tolerant coliforms (Most Probable Number - MPN: 0.0 MPN/g). The population of coagulase positive *staphylococci* was <1.0 x 10^2 CFU/g.

3.5. Real time quantitative PCR (RealT-qPCR) analysis

Although the culture-dependent plate count method is considered "gold standard" for evaluation of microorganisms viability (Champagne et al., 2011), it also has limitations as it only detects the cultivable fraction of microbial populations (Juste et al., 2008) and "viable, but non-cultivable" cells are missed (Achilleos and Berthier, 2013).

Thus, to better evaluate the behavior of the starter culture added, the RealT-qPCR assay was performed on the total DNA directly extracted from the samples collected during processing and from Mozzarella cheese samples (MCSTM5 and MCSJRP107), in different periods of storage. In all samples, *St. thermophilus* was successfully identified and quantified (Fig. 3). The RealT-qPCR became the most used method for quantification of microorganisms in complex environments such as foods (Juste et al., 2008), despite its inability to distinguish live and dead cells, and viable or none, since DNA from both celular states are amplified.

In this study, SYBR green fluorescent dye was used for RealT-qPCR analysis and good results were obtained, in terms of specificity, correlation coefficient and efficiency (date not shown). Moreover, the SYBR green preferential binding to specific DNA fragments in RealT-qPCR has been demonstrated in different researches (Miller et al., 2012; Bottari et al., 2013; Achilleos and Berthier, 2013; Ruggirello et al., 2014). The species-specific primers used to attach the conserved region of *pheS* gene were choose instead of 16S rRNA sequence mainly because the *pheS* gene has been reported as an useful alternative DNA target in discriminating different species of *Lactobacillus* (Achilleos and Berthier, 2013). Additionally, the *pheS* gene is present in a single copy, and therefore, it may be more suitable as target for an eventual quantification of the microbial species. Furthermore, primers directed toward the *pheS* gene exhibited a good level of specificity for LAB identification, being sensitive enough to detect even minor *St. thermophilus* amounts from whey starter (Bottari et al., 2013). Thus, a single peak was observed (50 pb size) on the amplicon melting curve, with melting temperature (Tm) about 75 °C.

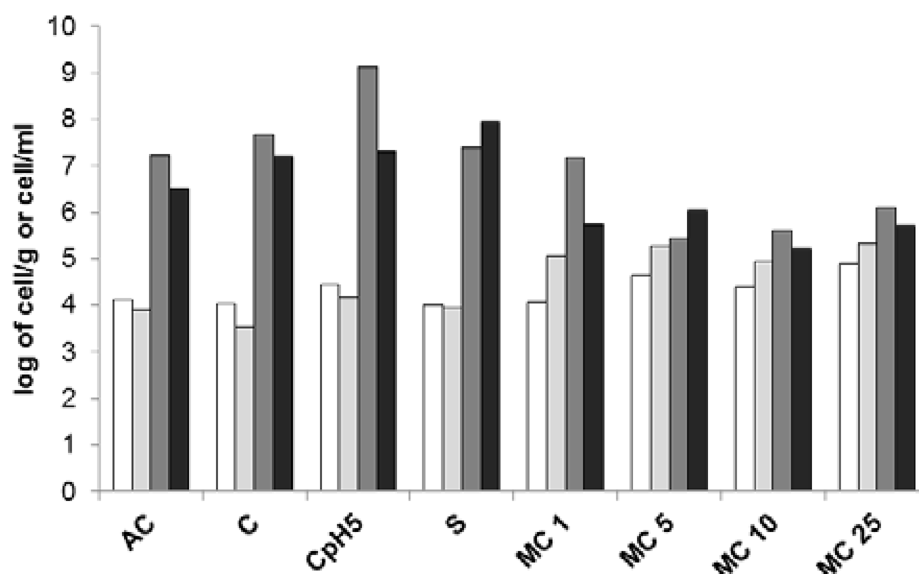


Fig. 3 Quantification of *St. thermophilus* by RealT-qPCR and plate counting in samples of milk after addition of culture (AC), curd (C), fermented curd ($CpH_{5.0}$), stretched curd (S), Mozzarella cheese samples after 1 (MC1), 5 (MC5), 10 (MC10) and 25 (MC25) days of storage. The RealT-qPCR by MCSTM5 is represented by white column and to MCSJRP107 by high gray column. The plate counting in M17 (Difco) at 42 °C by MCSTM5 is represented by dark gray column and to MCSJRP107 by black column. The results were expressed in log 10.

The *St. thermophilus* quantified by RealT-qPCR were compared with the cultivable *St. thermophilus* cell counts on M17 plates at 42 °C in samples collected during cheese making and storage period. In general, the population of *St. thermophilus* determined by the RealT-qPCR were lower (about 1.0 to 5.0 log cycles) than those obtained by the plate counting (Fig 3). The more discrepancy of the counting between the two methods was observed in samples collected during cheese making. It is also found higher mesophilic and thermophilic counting in M17 and MRS agar. However, it is important to highlight that these methods give different information about the cultures present in cheese. Plate counting enables to know about the viable and cultivable microbial populations in cheese, while the RealT-qPCR is often used to assessment the total DNA present in food matrixes because it detection thresholds is similar or higher than standard plate counts (Aparecida de Oliveira et al., 2010; Chen et al., 2010; Achilleos and Berthier, 2013; Martinez et al., 2015).

In our case, these data could be interpreted as a lack of selectivity of M17 agar, in which colony growth is not only related to *St. thermophilus*. The counting can be related to the presence of other LAB species, such as NSLAB, like *Enterococcus* sp., which is also cultivable in M17 at 42 °C and often isolated from buffalo Mozzarella cheese (De Angelis et al., 2008; Silva et al., 2015). In this context, probably the NSLAB counts were higher during

cheese making than in the storage period. Additionally, it is also interesting to note that *St. thermophilus* counts is higher in RealT-qPCR when NSLAB is lower in plate counts showing the competition between starter LAB (SLAB) and NSLAB. Moreover, in general, the behavior and evolution of counts to MCSTM5 and MCSJRP107 was similar in both methods.

On the other hand, considering this hypothesis, the characteristic of the cheese during storage is directly related to the presence of the starter *St. thermophilus* culture, since the RealT-qPCR and plate counting methods showed similar counts. Additionally, the SJRP107 was previously isolated from buffalo Mozzarella cheese during refrigerated storage (data not shown).

Furthermore, it is important to point out that some aspects during analysis can affect the RealT-qPCR results, such as quality of nucleic acid extracts, specificity of viability dyes for live and dead cells, dye incubation conditions, photoactivation, target gene, species-specific differences in viability PCR, and matrix characteristics. In complex matrix, such as cheese, different factors including ionic compounds, water activity, pH, and a high number of dead cells have the potential to interfere with RealT-qPCR results (Postollec et al., 2011; Fittipaldi et al., 2012; Gianfrancesch et al., 2014). Therefore, it is possible that RealT-qPCR method cannot be used to accurately quantify *St. thermophilus* in cheese matrix because of such difficulties, including the first step in the analysis, the lyse of the cells for nucleic acid extraction. Despite the extracted DNAs have shown good results (ratio of 1.60-1.90 at wavelength 260/280), some authors show the difficulty for the bacterial DNA extraction in soft cheese matrix and for RealT-PCR amplification due to its high fat and protein content (Gianfrancesch et al., 2014). Thus, further studies are necessary to obtain more efficient quantification of *St. thermophilus* using RealT-qPCR from cheese samples to overcome these limits.

4. CONCLUSIONS

The autochthonous *St. thermophilus* SJRP107 culture was considered safe and showed high potential technological of application in buffalo Mozzarella cheese production. Their application as starter culture in Mozzarella cheese resulted in texture, chemical and proteolytic properties similar to that manufactured with commercial culture. In general, the LAB plate counting showed the same tendency for both treatments. Additionally, the MCSJRP107 showed more stable texture than MCSTM5 during of storage period. The RealT-

qPCR method indicated the presence of *St. thermophilus* starter culture until the end of storage, demonstrating their robustness for cheese production. Additional studies will be carried out for the commercial application of this strain.

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Capítulo V

CAPÍTULO V

CONSIDERAÇÕES FINAIS

Os resultados obtidos neste estudo mostram a biodiversidade e a dinâmica das bactérias acidoláticas autóctones presentes durante as etapas do processo de produção e período de estocagem do queijo Muçarela de búfala, além de apresentar novas culturas láticas com características tecnológicas interessantes para a aplicação industrial.

- As BAL isoladas foram identificadas como: *Enterocccus faecalis*, *Lactococcus. garvieae*, *Lactobacillus helveticus*, *Lactococcus lactis*, *Streptococcus thermophilus*, *Leuconostoc citreum*, *Lactobacillus delbrueckii* subsp. *bulgaricus, Enterococcus sp.*, *Lactobacillus fermentum*, *Lactobacillus casei e Leuconostoc. mesenteroides*. Com exceção do *Lc. citreum*, as BAL isoladas neste estudo são tipicamente encontradas no tradicional queijo de origem italiana. Os resultados mostraram a dinâmica das BAL representativas nas diferentes etapas de processamento e período de estocagem do queijo Muçarela.

- A capacidade de utilizar o citrato em meio diferencial foi observada por todas as culturas de *Lc. mesenteroides*, *Lc. citreum*, *L. lactis* e *Lb. fermentum* avaliadas, bem como, por algumas cepas de *Lb. casei*. A maioria das cepas produziu proteases extracelulares e foram capazes de reduzir o pH do leite para $\leq$ 5.0 no final da fermentação, bem como produzir altas concentrações de compostos orgânicos. A produção dos ácidos orgânicos e acetoína foi espécie-dependente.

- Dentre as quatro culturas avaliadas, a cultura de *St. thermophilus* SJRP107 foi selecionada como cultura *starter* para a fabricação do queijo Muçarela de búfala sendo considerada segura e apresentando boa atividade acidificante. A sua aplicação como cultura *starter* no queijo Muçarela de búfala resultou em composição química, textura e proteólise semelhante ao queijo produzido usando cultura comercial. Além disso, a textura do queijo MCSJRP107 (produzido com a cultura autóctone) se mostrou mais estável durante o período de estocagem do que o queijo MCSTM5 (produzido com a

cultura comercial). Em geral, as contagens das BAL durante o processamento foram maiores que durante o período de estocagem. O método Real Time-qPCR indicou a presença de *St. thermophilus* até ao final do período de estocagem, demonstrando a sua robustez para a produção do queijo.