UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM MICROBIOLOGIA
AGRÍCOLA E DO AMBIENTE

AVALIAÇÃO DA ATIVIDADE PROTEOLÍTICA E FORMAÇÃO DE BIOFILMES POR BACTÉRIAS PSICROTRÓFICAS ISOLADAS DE LEITE CRÚ DE BÚFALA REFRIGERADO

MARCIÉLE BOGO

Orientador (a): Profª. Drª. Amanda de Souza da Motta
Co-Orientador (a): Profª. Drª. Ana Paula Guedes Frazzon

Porto Alegre
Março/2016

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM MICROBIOLOGIA
AGRÍCOLA E DO AMBIENTE

AVALIAÇÃO DA ATIVIDADE PROTEOLÍTICA E FORMAÇÃO DE BIOFILMES POR BACTÉRIAS PSICROTRÓFICAS ISOLADAS DE LEITE CRÚ DE BÚFALA REFRIGERADO

MARCIÉLE BOGO
Bióloga (UNOESC)

Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Microbiologia Agrícola e do Ambiente da Universidade Federal do Rio Grande do Sul, como parte dos requisitos necessários à obtenção do título de Mestre em Microbiologia Agrícola e do Ambiente.

Orientador(a): Profª. Drª. Amanda de Souza da Motta
Co-orientador(a): Profª. Drª. Ana Paula Guedes Frazzon

Porto Alegre, Rio Grande do Sul – Brasil
Março/2016

CIP - Catalogação na Publicação

BOGO, MARCIÉLE
AVALIAÇÃO DA ATIVIDADE PROTEOLÍTICA E FORMAÇÃO DE BIOFILMES POR BACTÉRIAS PSICROTRÓFICAS ISOLADAS DE LEITE CRÚ DE BÚFALA REFRIGERADO / MARCIÉLE BOGO. -- 2016.
123 f.

Orientador: AMANDA DE SOUZA DA MOTTA.
Coorientador: ANA PAULA GUEDES FRAZZON.

Dissertação (Mestrado) -- Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Programa de Pós-Graduação em Microbiologia Agrícola e do Ambiente, Porto Alegre, BR-RS, 2016.

1. BACTÉRIAS PSICROTRÓFICAS. 2. CONTAMINAÇÃO. 3. DETERIORAÇÃO. 4. BIOFILME. 5. LEITE DE BÚFALA. I. DE SOUZA DA MOTTA, AMANDA , orient. II. GUEDES FRAZZON, ANA PAULA, coorient. III. Titulo.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

“Tudo o que vive, não vive sozinho, nem por si”

William Blake

AGRADECIMENTOS

Agradeço primeiramente a Deus, pela proteção, amparo e por iluminar meu caminho nos momentos difíceis de minha jornada, dando-me forças para vencer.

À minha família, em especial, aos meus pais **Vicente e Guimar**, pelo incentivo e apoio nessa jornada em busca do conhecimento.

Ao meu marido **Mateus**, que além de ser companheiro sempre acreditou em mim e esteve ao meu lado, mesmo em tempos difíceis. Muito obrigada pela companhia nas madrugadas na rodoviária, pela confiança, apoio e pelos conselhos fortalecedores.

À minha querida orientadora, **Amanda Motta**, que me acolheu na UFRGS de braços abertos. Agradeço pela sua disponibilidade em repassar o seu conhecimento, sendo sempre acessível e disposta a ajudar. Sou grata também pela paciência e pelos conselhos nos momentos quando me sentia sozinha.

À minha co-orientadora **Ana Paula Frazzon**, que com seu bom humor, sempre compartilhou suas ideias e auxiliou para a efetivação desse trabalho.

Aos professores do ppgmaa, que pelos ensinamentos que colaboraram para a concretização de mais uma etapa da minha vida.

Aos colegas e amigos que fiz no Lab 165, **Thais**, **Stefano**, **Géssica**, **Karine**, **Álvaro**, **Ingrid**, **Maya**, **Helena, Milena** e **Maria Rita**, pela convivência agradável, companheirismo e pelas trocas de conhecimento. Vou lembrar sempre das nossas risadas, dos desenho e joguinhos no quadro nos momentos de folga e das saídas. Obrigada por fazer essa etapa passar mais suavemente.

As minhas amigas **Michelly** e **Mônica**, pelo incentivo no início e o apoio no decorrer desse trabalho. E principalmente, pela amizade mantida mesmo com a distância.

Aos demais colegas de aula do ppgmaa, pela troca de informações e conhecimentos.

Aos demais laboratórios que deram suporte, emprestando equipamentos e reagentes, obrigado por serem acessíveis e cordiais.

E a todos que direta ou indiretamente contribuíram de alguma forma para a realização do trabalho.

Ao CNPq pelo apoio financeiro.

AVALIAÇÃO DA ATIVIDADE PROTEOLÍTICA E FORMAÇÃO DE BIOFILMES POR BACTÉRIAS PSICROTRÓFICAS ISOLADAS DE LEITE CRÚ DE BÚFALA REFRIGERADO

Autor: Marciéle Bogo

Orientadora: Profª. Drª. Amanda de Souza da Motta

Co-Orientadora: Profª. Drª. Ana Paula Guedes Frazzon

RESUMO

Bactérias psicrotróficas representam um impacto negativo para a indústria de lácteos, por serem microrganismos que desenvolvem-se em temperaturas baixas, apresentam potencial para a produção de enzimas hidrolíticas e formação de biofilmes. Este estudo teve o objetivo de avaliar a capacidade de produção de enzimas hidrolíticas termoresistentes e verificar o potencial de formação de biofilmes por 21 bactérias psicrotróficas isoladas de leite crú de búfala refrigerado. Esses isolados foram inicialmente identificados como pertencentes aos gêneros *Pseudomonas*, *Chryseobacterium*, *Enterobacter*, *Burkholderia*, *Acinetobacter* e *Oligella*. Dos 21 isolados, 42,85 % produziram enzimas lipolíticas, 33,33 % foram produtores de lecitinase e todos os isolados produziram proteólise em ágar leite bovino e bubalino, sendo que 5 isolados foram produtores de enzimas proteolíticas termoresistentes. Quando a formação de biofilme, 16 bactérias foram formadoras de biofilme a 7℃ e 20 apresentaram este potencial à 2 3 ℃. À 37℃, 17 isolados foram considerados fracos formadores de biofilme. Na presença de resíduos de leite bubalino e bovino, também encontramos isolados formadores de biofilmes. Na avaliação da formação de biofilmes sobre as superfícies de aço inoxidável e polipropileno, dois isolados de *P. fluorescens* produziram os biofilmes, inclusive quando avaliados em co-cultivo com *Staphylococcus aureus* A710^{-2}. Na avaliação da proteólise dentro do biofilme os isolados de *P*. *fluorescens* apresentaram este potencial, porém não em níveis maiores que as mesmas células planctônicas. Os resultados deste estudo mostram que as bactérias psicrotróficas têm o potencial de deterioração e formação de biofilmes. Neste sentido destaca-se a importância para a preocupação com a adoção de boas práticas na produção desta matéria-prima, considerando que 100% do leite bubalino, comercializado no Rio Grande do Sul, são destinados à elaboração de produtos derivados.

Palavras-chave: Bactérias psicrotróficas. Contaminação. Deterioração. Biofilmes. Leite de búfala.

[1]Dissertação de Mestrado em Microbiologia Agrícola e do Ambiente – Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil. (129 p.) março, 2016.

PROTEOLYTIC ACTIVITY AND ASSESSMENT OF BIOFILM FORMATION BY PSYCHROTROPHIC BACTERIA ISOLATED FROM REFRIGERATED BUFFALO MILK RAW

Author: Marciéle Bogo[1]

Advisor: Profa. Dra. Amanda de Souza da Motta

Co-advisor: Profa. Dra. Ana Paula Guedes Frazzon

ABSTRACT

Psychrotrophic bacteria represent a negative impact on the dairy industry, because they are microorganisms that develop in low temperatures, have the potential for production of hydrolytic enzymes and biofilm formation. This study aimed to evaluate the enzyme production capacity hydrolytic heat-resistant and verify the potential of biofilm formation by 21 isolated psychrotrophic bacteria isolated from refrigerated buffalo raw milk. These isolates were initially identified as belonging to the genera *Pseudomonas*, *Chryseobacterium*, *Enterobacter*, *Burkholderia, Acinetobacter* and *Oligella*. Of the 21 isolates, 42,85% produced lipolytic enzymes, 33.33% were lecithinase producers and all isolates were produced proteolysis on bovine and buffalo milk agar, being that 5 isolates were producers of heat-resistant proteolytic enzymes. When the biofilm formation was evaluated, 16 biofilm forming bacteria at 7℃ and 20 showed this potential at 23 ℃. At 37 ℃ , 17 isolates were considered weak biofilm formers. In the presence of residue buffalo and bovine milk also found isolated forming biofilms. In the evaluation of the formation of biofilms on the surfaces of stainless steel and polypropylene, two isolates of *P. fluorescens* produced biofilms, also when evaluated in co-culture with *Staphylococcus aureus* A710^{-2}. In the evaluation of proteolysis within of biofilm, *P. fluorescens* isolates showed this potential, but not at levels greater than the same planktonic cells. The results of this study show that psychotrophic bacteria have the potential to spoilage and formation of biofilms. In this regard highlights the importance for concern with the adoption of best practices in the production of this raw material, considering that 100% of buffalo milk, comercialized in Rio Grande do Sul/Brazil, are intended for the manufacture of dairy products.

Keywords: Psychrotrophic Bacteria. Contamination. Deterioration. Biofilms. Buffalo Milk

[1]Master of Science Thesis in Agricultural and Environmental Microbiology – Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. (129 p.), march, 2016.

SUMÁRIO

RELAÇÃO DE TABELAS

RELAÇÃO DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

%: percentual
(-): negativo
(+): positivo
(v / v): volume por volume
<: menor
±: mais ou menos
®: marca registrada
℃: graus Celsius
µg: microgramas
µl: microlitros
µM: micromolar
ATCC: American Type Culture Collection
CFU / cm^2: colony forming unit por centímetros quadrados
CFU / ml: colony forming unit por mililitro
CLSI: Clinical and Laboratory Standards Institute
cm^2: centímetros quadrados
DNA: desoxirribonucleico ácido
EPS: exopolissacarídeo
g L^{-1}: gramas por litro
Log: logaritmo
OD: densidade ótica
M: molar
mg / ml: miligrama por mililitro
mg: miligramas
ml: mililitros
mm: milimetros
nm: nanometros
pb: pares de base
pH: potencial de Hidrogenio
pM: picomolar
PU / cell: picos de Unidades por celulas
Rpm: rotação por minuto
SDS: dodecilsulfato de sódio
TM: marca ainda não registrada
U: Unidade
UHT: ultra high temperature

1. INTRODUÇÃO

A composição do leite faz com que ele tenha destaque como um dos alimentos mais nutritivos para o ser humano. O leite de búfala apresenta características que o diferenciam de qualquer outro tipo de leite. Quando comparado com leite bovino, o leite de búfala tem um teor mais elevado de proteínas, gorduras, lactose e minerais, sendo um ingrediente adequado para a fabricação de uma grande variedade de produtos lácteos, tais como queijo, manteiga, creme de leite, requeijão e iogurte. A produção de leite bubalino no Brasil tem aumentado nas últimas décadas, devido a procura por derivados, chagando a produção de 92 milhões de litros por ano.

Como em qualquer leite cru, os microrganismos podem multiplicar-se rapidamente devido ao seu alto teor em nutrientes. A ocorrência de altos níveis de bactérias no leite, podem causar alterações químicas, tais como a degradação de gorduras, de proteínas ou de carboidratos, podendo tornar o produto impróprio para o consumo e industrialização e apresentar um potencial perigo à saúde.

Mesmo em condições de refrigeração, o leite pode ser facilmente deteriorado, servindo para a proliferação de um grande número de bactérias. A ação deteriorante em condições de refrigeração é provocada por bactérias psicrotróficas, que inclui espécies que apresentam tempo de geração curto e crescem em temperaturas baixas de refrigeração. São em sua maioria Gram-negativas, provenientes do ambiente e equipamentos de ordenha.

A maioria das bactérias psicrotróficas não sobrevive à temperatura de pasteurização, porém, produzem uma variedade de enzimas extracelulares, muitas delas termoresistentes, principalmente proteases e lipases que hidrolisam respectivamente proteína e gordura do leite e derivados, o que gera um grande problema para a indústria de produtos lácticos.

Outra característica relevante das bactérias psicrotróficas é a capacidade de adesão e formação de biofilmes. As falhas nos procedimentos de higienização permitem que os resíduos de leite fiquem aderidos a superfícies e equipamentos sendo uma potencial fonte de contaminação. Sob essas condições, as bactérias aderem-se, interagem com a superfície e iniciam o crescimento celular, dando origem a um aglomerado de células que agrega nutrientes, resíduos e outras

bactérias, formando a estrutura denominada biofilme. Nessa situação, as bactérias estão mais resistentes à ação de agentes químicos e físicos usados nos procedimentos de higienização.

O leite ou produto derivado pode ser contaminado por bactérias patogênicas ou deteriorantes indesejáveis provenientes dos biofilmes, e isso pode acarretar problemas graves e perdas econômicas devido à diminuição de vida de prateleira do produto, além de ser considerada uma fonte crônica de contaminação por patógenos alimentares.

As avaliações da presença de bactérias psicrotróficas no leite cru de búfala são escassas. Neste sentido, torna-se importante identificar isolados com potencial deteriorante e investigar a capacidade de formação de biofilme, uma vez que esses fatores podem resultar em graves problemas, comprometendo o rendimento e a qualidade do alimento, apresentando riscos à saúde do consumidor e também ocasionando prejuízos financeiros à indústria.

2 OBJETIVOS

2.1 Objetivo Geral

Este trabalho propõe avaliar a atividade proteolítica e a capacidade de formação de biofilmes por bactérias psicrotróficas isoladas de leite cru de búfala refrigerado.

2.2. Objetivos Específicos

- Avaliar a produção de enzimas extracelulares - proteolítica, lipolítica e lecitinase - pelas bactérias psicrotróficas isoladas de leite cru de búfala refrigerado.
- Verificar a suscetibilidade das bactérias psicrotrófica isoladas de leite cru refrigerado de búfala frente a antimicrobianos.
- Avaliar a habilidade da produção de enzimas proteolíticas termoresistentes e sua estabilidade no leite cru refrigerado bubalino e bovino.
- Avaliar o potencial de formação de biofilme de bactérias psicrotróficas.
- Avaliar o potencial de formação de biofilmes na presença de resíduos de leite bovino e bubalino e em diferentes superfícies: aço inoxidável e polipropileno.
- Avaliar a produção de enzimas proteolíticas dentro de biofilme pelas bactérias psicrotróficas isoladas de leite cru de búfala refrigerado.

3. REVISÃO BIBLIOGRÁFICA

3.1 Leite Bubalino

O búfalo (*Bubalus bubalis*) é criado em vários países asiáticos para a produção leiteira. Essa prática vem se difundindo e aumentando para os demais continentes devido à aceitação do consumo desse leite e de seus produtos derivados, tornando-se o segundo leite mais consumido no mundo, ficando somente atrás do leite bovino (HAN et al., 2012).

No Brasil, tem ocorrido um aumento crescente na criação de búfalos, em decorrência da adaptabilidade e rusticidade (FAO, 2013) e devido às características físico-químicas peculiares do seu leite (BARRETO et al., 2010).

De acordo com dados publicados em 2010 pelo MAPA - Ministério da Agricultura, Pecuária e Abastecimento (BRASIL, 2010), são apresentados valores de um efetivo de 1,5 bilhões de búfalos, sendo estes distribuídos pelas cinco regiões do país, respectivamente nas seguintes quantidades e proporções: Norte: 820.295 (67,93%); Sudeste: 115.404 (9,55%); Nordeste: 112.053 (9,28%); Sul: 105.264 (8,71%) e Centro-Oeste: 54.445 (4,50%); destacando-se as seguintes raças: Murrah; Jafarabadi e suas cruzas; Mediterrâneo; e Carabao.

De acordo com Damé et al. (2010), a produção de leite de búfala no Sul do Brasil é de 130 mil litros por ano, correspondendo a 8,71% do total da produção no país.

O leite de búfala é uma fonte rica de grandes componentes que são essenciais para fornecer as exigências nutricionais ao corpo humano (AHMAD et al., 2013). Seu sabor é peculiar, ligeiramente adocicado e mais branco do que o leite bovino, devido à ausência de β-caroteno, ainda, contém níveis elevados de sólidos totais, proteína bruta, gordura, cálcio, fósforo e ligeiramente maior teor de lactose em comparação com o do leite bovino (VERRUMA & SALGADO, 1994; MINARD et al., 2010; HAN et al., 2012; AHMAD et al., 2013). Além de suas vantagens nutricionais, um estudo realizado por Sheehan et al. (2009), afirma que indivíduos com alergias ao leite bovino seriam capazes de tolerar leite bubalino.

Em geral, o leite bubalino é mais concentrado do que o leite bovino apresentando desse modo, menos água e mais matéria seca (FARIA et al., 2008; SOARES et al., 2013). Possui teores de proteínas entre 3,5 e 4,5%, gorduras entre 6

e 7,5%, lactose entre 4,5 e 5,5% e sólidos totais de 17% (COELHO et al., 2004; SOARES et al., 2013; DE LIMA et al., 2014).

Em especial, o alto nível de sólidos totais do leite de búfala torna-o ideal para a conversão em produtos lácteos, como queijo, requeijão, iogurte, sorvete, manteiga, mussarela, entre outros, que estão se tornando cada vez mais populares devido ao rico conteúdo nutricional (JAYAMANNE & ADAMS, 2004; HAN et al., 2012).

O consumo do leite de búfala em seu estado natural no Brasil ainda não é difundido. A grande importância desse alimento está na sua transformação em derivados, em sua totalidade, destinado à produção de *mozzarella* e manteiga (ANDRIGHETTO, 2005; ARAÚJO et al., 2012), uma vez que sua composição peculiar possibilita um alto rendimento industrial (OLIVIERI, 2004; HUSSAIN et al., 2012).

Apesar do aumento da produção do leite bubalino e de sua inserção significativa no mercado, é importante salientar a falta de uma legislação específica no Brasil para determinar o padrão de identidade físico-química e qualidade microbiológica do leite bubalino. Somente o Estado de São Paulo (SÃO PAULO, 1994) possui uma legislação para alguns parâmetros de qualidade.

De acordo com estudos, após a ordenha, o leite pode apresentar uma grande quantidade de bactérias, tais como *Escherichia coli*, *Staphylococcus aureus*, *S. lentus*, *Citrobacter*, *Enterobacter cloacae*, *Listeria* sp., *Enterococcus faecium* e *Pseudomonas* sp. (HAN et al., 2007; GÜRLER et al., 2013; LAMAGNA et al., 2015; HASHM & SALEEM, 2015).

3.2 Bactérias Psicrotróficas no Leite

O leite cru contém uma grande variedade de bactérias psicrotróficas e mesófilas do ambiente da fazenda de gado leiteiro (TEH et al., 2011). O uso da refrigeração nas fazendas e nas indústrias de processamento lácteo, para o armazenamento do leite cru, possibilita o controle de microrganismos aeróbios mesófilos e coliformes totais (BRASIL, 2011). No entanto, quando a contaminação inicial do leite é grande e ocorre o armazenamento por períodos prolongados, pode resultar na queda da qualidade dos produtos lácteos, devido ao crescimento de

bactérias psicrotróficas (DE JONGHE et al., 2011; SAMARZIJA et al., 2012; PERIN et al., 2012).

Bactérias psicrotróficas são definidas por possuírem capacidade de crescimento em baixas temperaturas (4-7℃), independente de sua temperatura ótima de crescimento, a qual pode ser de 20℃ a 30° C. Estes microrganismos podem ser bastonetes, cocos, formadores ou não de esporos, Gram-negativos, aeróbios ou anaeróbios (SORHAUG & STEPANIAK, 1997; POTHAKOS et al., 2012; VITHANAGE et al., 2014). O grupo das bactérias psicrotróficas são representados principalmente por *Pseudomonas* sp.*, Staphylococcus* sp.*, Listeria* sp.*, Aeromonas* sp.*, Serratia* sp.*, Chromobacterium* sp. e *Microbacterium* sp. (ENEROTH et al., 2000; TEH et al., 2011), sendo que o grupo de bactérias Gram-negativas é mais o prevalente entre as bactérias psicrotróficas, como é demonstrado em muitos estudos (PINTO et al., 2006; ERCOLINI et al., 2009; TEH et al., 2012; MACHADO et al., 2013; QUIGLEY et al., 2013; NEUBECK et al., 2015; BAUR et al., 2015; MCINNIS et al., 2015).

Embora as bactérias psicrotróficas representem menos que 10% da microbiota total do leite fresco obtido em condições higiênico-sanitárias satisfatórias, podem chegar a 75% sob condições inadequadas (NIELSEN, 2002; PINTO et al., 2006).

A contaminação dos produtos lácteos por bactérias psicrotróficas pode originar-se do suprimento de água de qualidade inadequada, deficiências de procedimentos de higiene e mastite (MILLOGO et al., 2010). Dessa forma, a presença de microrganismos psicrotróficos no leite depende da eficiência com que os procedimentos de limpeza e sanitização das superfícies que entram em contato com o leite são efetuados, assim como da higiene do ambiente, da temperatura de manutenção e do tempo decorrente até o seu processamento (HOLM et al., 2004; ARCURI et al., 2008; SWAI & SCHOONMAN, 2011; ALVES et al., 2014).

Uma importante característica dos psicrotróficos encontrados no leite e produtos derivados é a sua capacidade de síntese, durante a fase logarítmica, de enzimas extracelulares que degradam os componentes do leite (VITHANAGE et al., 2014). Ainda que, a maioria das bactérias presentes no leite cru sejam eliminadas durante o processo de pasteurização ou por outros tratamentos térmicos, estes têm pouco efeito sobre a atividade das enzimas termoresistentes produzidas por estes microrganismos (NÖRNBERG et al., 2010).

Devido à característica de rápida multiplicação no leite e à produção de enzimas extracelulares que permanecem após a pasteurização, observa-se a importância de manter a qualidade do leite cru durante a ordenha e transporte, com procedimentos de limpeza e sanitização adequados, para garantir uma boa qualidade de produtos lácteos finais (TEH et al., 2012).

Apesar da importância dos psicrotróficos, o Ministério da Agricultura não estipula um padrão de qualidade e identidade do leite, baseado na contagem de unidade formadora de colônias (UFC) destes microrganismos. No Brasil, não existe uma regulamentação sobre a qualidade microbiológica de bactérias psicrotróficas do leite *in natura*. No entanto, conforme Pinto et al. (2006) não é recomendável a fabricação de produtos lácteos com leite cru com contagem de psicrotróficos superior a 6,7 log UFC / mL, devido a sua capacidade de multiplicação em condições de refrigeração.

3.3 Enzimas Bacterianas no Leite

As enzimas indesejáveis podem diminuir o valor de produtos alimentares. Na indústria de láteos, essas enzimas indesejáveis geralmente incluem enzimas provenientes do animal leiteiro e enzimas microbianas produzidas pelas bactérias associados com o ambiente de ordenha (TEH et al., 2014).

As enzimas extracelulares indesejáveis são produzidas por bactérias contaminantes durante a fase exponencial e estacionária do crescimento bacteriano (HADDADI et al., 2005; VITHANAGE et al., 2014). É um processo complexo influenciado pela concentração de oxigênio, *quorum sensing*, temperatura, teor de ferro e variação de fase de crescimento (BUCHON et al., 2000; CHEN et al., 2003; NICODÈME et al., 2005; MARCHAND et al., 2009).

A produção de enzimas extracelulares se torna um grande problema na indústria de laticínios, pois a maioria das enzimas resiste aos processos de tratamento térmico, como *Ultra High Temperature* (UHT) (BAGLINIÈRE et al., 2013), pasteurização lenta (63,5 ℃ por 30 minutos) (TEH et al., 2011) e pasteurização rápida (76℃ por 30 segundos) (NÖRNBERG et al., 2010), ao contrário do que ocorre com a maioria das bactérias contaminantes que são eliminadas por esses processos térmicos (TONDO & BARTZ, 2012).

Das principais enzimas extracelulares produzidas por bactérias contaminantes, destacam-se enzimas proteolíticas e lipolíticas. Estas degradam os principais componentes do leite, que são respectivamente proteínas e lipídios (Tabela 1) (DEETH, 2006; MARTINS et al., 2006; NÖRNBERG et al., 2011).

As lipases são enzimas que catalisam a hidrólise dos triglicerídeos, os principais componentes lipídicos de leite. Os produtos da reação são os ácidos graxos livres e glicerídeos parciais (mono e diglicerídeos) e, em alguns casos, o glicerol (DEETH, 2006; TEH at al., 2013).

A atividade das enzimas lipolíticas produz um sabor rançoso e a perda de propriedades funcionais no leite, sendo uma constante preocupação para a indústria de alimentos (BEKKER et al., 2016). Apesar das enzimas presentes no leite processado e nos produtos lácteos poderem estar em baixas concentrações, ao longo do tempo, estas enzimas passam a alterar as propriedades físico-químicas do produto lácteo (CHEN et al., 2011).

A enzima proteolítica provoca a coagulação, gelificação e deterioração das características sensoriais do leite. Estas enzimas apresentam a capacidade de degradar caseína, principal proteína do leite, promovendo a agregação de micelas, consequentemente promovendo a perda das propriedades funcionais do produto lácteo (BUTTON et al., 2011; TEH et al., 2011).

Os mecanismos envolvidos no fenômeno da coagulação promovidos pela participação das enzimas proteolíticas, compreendem basicamente, na alteração de proteínas do leite, associação e dissociação de íons de cálcio, formação e dissociação do complexo κ-caseína com proteínas do soro do leite (CHEN et al., 2003). A maioria destas proteases têm a capacidade de hidrolisar a k-caseína a para-k-caseína, e desestabilizar a micela de caseína, o que por sua vez provoca a coagulação do leite (FAIRBAIRN & LAW, 1986; ZHANG & LV, 2014).

De acordo com Nörnberg et al. (2009), não existe uma correlação direta entre as contagens de psicrotróficos e a atividade proteolítica, já que possivelmente a composição da microbiota de bactérias psicrotróficas presentes no leite cru influencia no desenvolvimento da proteólise. No entanto, quando em experimento *in vitro*, as bactérias psicrotróficas atingem um nível de células 10^6 UFC / ml, a produção de proteases por tais microrganismos é capaz de degradar quantidades consideráveis de caseína (NÖRBERG et al., 2011).

Tabela 1. Efeito do crescimento de microrganismos psicrotróficos sob a qualidade dos produtos lácteos.

Produto	**Psicrotróficos no leite cru (log UFC / ml)**	**Efeitos na qualidade**
Leite UHT	5,9	Gelificação após 20 semanas
	6,9 -7,2	Gelificação após 2 a 10 semanas, odor estranho, sabor amargo
Leite em pó	6,3 - 7	Redução da estabilidade ao calor, aumento da formação de espumas em leite reconstituído
Leite pasteurizado	5,5	Sabor inferior, quando comparado com o leite pasteurizado fresco
	7 - 8	Menor vida de prateleira Depósito no trocador placas
Queijo massa dura	6,5 – 7,5	Rancidez
	7,5 – 8,3	Sabor diferente, predomínio de gosto ranço e sabão e menor rendimento
Queijo Cottage	5 – 7,8	Gosto amargo
Manteiga	Não determinado	Rápido desenvolvimento de rancidez
Iogurte	7,6 – 7,8	Gosto amargo, sabor adocicado, dependendo da microbiota específica

Fonte: SORHAUG & STEPANIAK, 1997.

Conforme o estudo de Teh et al. (2012) e Teh et al. (2013), as atividades enzimáticas proteolítica e lipolítica podem ser produzidas em biofilmes sobre as superfícies que entram em contato com o leite cru. Conforme resultados destes estudos, houve uma maior atividade proteolítica e lipolítica dentro de biofilmes do que nas suas culturas planctônicas. Isso tem importância prática para a indústria de laticínios, porque as bactérias presentes nos biofilmes em superfícies podem ser uma fonte de lipases e proteases termoresistentes. Isso mais uma vez destaca a

necessidade de contínua limpeza e sanitização de equipamentos e superfície que entram em contanto com o leite cru.

3.4 Biofilmes Microbianos

Muitas bactérias psicrotróficas podem sobreviver aderidas em superfícies de contato com líquidos formando agregados de células denominados biofilmes. A presença de biofilme na indústria de lácteos representa um risco à saúde do consumidor devido à probabilidade de disseminação de bactérias patogênicas e suas toxinas, e pode ocasionar prejuízos financeiros em virtude da diminuição da vida de prateleira dos produtos, provocados pelas bactérias deteriorantes (SOFOS & GEORNARAS, 2010; BURGESS et al., 2010; CARPENTIER & CERF, 2011).

De acordo com Abee et al. (2011), biofilmes são agregados de células microbianas embebidos em uma matriz polimérica ligados a uma superfície biótica ou abiótica. Conforme Teh et al. (2011), bactérias podem criar um microambiente que aumenta a sobrevivência microbiana sobre as superfícies. Resíduos do leite sobre as superfícies podem fornecer nutrientes para as bactérias sobreviverem e proliferarem, sobretudo quando as condições de crescimento são adequadas.

O desenvolvimento do biofilme pode ser dividido em cinco fases conforme pode ser visualizado na Figura 1. A formação do biofilme inicia com a junção de células bacterianas com moléculas orgânicas e inorgânicas depositadas na superfície biótica ou abiótica (BRIDIER et al., 2015).

A superfície da maioria das células bacterianas é carregada negativamente, esta carga negativa, para algumas superfícies de aderência, é adversa à aderência de bactérias, devido à força de repulsão eletrostática. No entanto, a superfície das células bacterianas, devido à hidrofobicidade, conta com estruturas externas como fímbrias, flagelos e lipopolissacarídeo (LPS) que formam uma ponte entre as células bacterianas e o substrato presentes nas superfícies de contato gerando uma associação mais consistente (TAKAHASHI et al., 2010; VAN HOUDT & MICHIELS, 2010; ABEE et al., 2011).

Após da adesão inicial, as células bacterianas multiplicam-se formando microcolônias e iniciam a secreção da matriz de exopolissacarídeo, constituindo um ambiente protegido de adversidades e favorecendo a incorporação de novas células microbianas e também de nutrientes (TOYOFUKU et al., 2012).

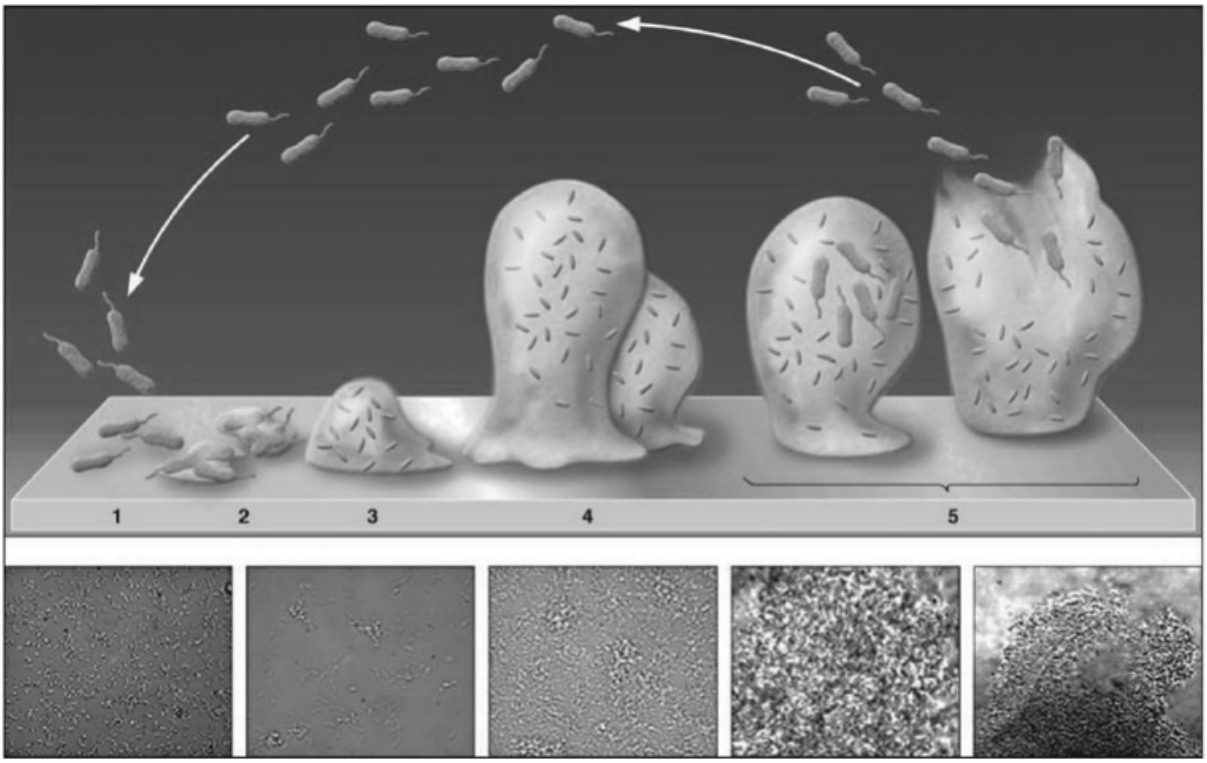

Figura 1. Estágios de desenvolvimento de biofilme bacteriano. Fase 1: A fixação inicial de células à superfície. Fase 2: Produção de exopolissacarídeo. Fase 3: Desenvolvimento inicial da arquitetura biofilme. Fase 4: Maturação da arquitetura do biofilme. Fase 5: Dispersão de células individuais do biofilme. Os painéis inferiores mostram cada uma das 5 fases de desenvolvimento representado por uma fotomicrografia de *P. aeruginosa* quando cultivada sob condições de contínuo-fluxo em um substrato de vidro.
Fonte: Stoodley et al. (2002).

A matriz de exopolissacarídeo que envolve as células microbianas é composta por uma série de componentes, como ácidos nucleicos, polissacarídeos, lipídeos e proteínas (Figura 2) (SHI & ZHU, 2009; TOYOFUKU et al., 2012). O aumento da densidade populacional, a grande produção e deposição de exopolissacarídeo e, também, aumento da espessura, provoca no biofilme a sua maturação (CHENG et al., 2007).

O biofilme maduro é caracterizado por uma arquitetura complexa que inclui canais e poros, no qual permitem a passagem de substâncias para dentro e para fora do biofilme (FLEMMING & WINGENDER, 2010), que se acredita ser

controlada pelo mecanismo de sinalização de célula-a-célula: *quorum-sensing* (SOHEILI et al., 2015).

Figura 2. Composição da matriz de exopolissacarídeo bacteriana.
Fonte: TOYOFUKU et al. (2012).

Após a maturação do biofilme, devido ao metabolismo, ocorre o aumento da concentração de moléculas que são responsáveis pela liberação de enzimas que degradam a matriz polimérica. Essas alterações no biofilme provocam a subsequente liberação de células individuais ou agregadas de um biofilme (BEHLAU & GILMORE, 2010).

As células desprendidas se apresentam móveis e assemelham-se às células planctônicas, podendo disseminar uma contaminação no alimento ou formar um novo biofilme na linha de produção (PALMER et al., 2007).

Conforme o estudo de Teh et al. (2012), o número de bactérias encontradas normalmente formando biofilmes varia de10^4 a 10^8 UFC / cm^2.

De acordo com o estudo de Morita et al. (2011), as bactérias são capazes de formar biofilmes em muitas superfícies abióticas, tais como plástico, borracha, vidro, cimento e aço inoxidável. Ainda, propriedades da superfície de fixação (por exemplo, rugosidade, estabilidade físico-química, resistência à corrosão) também são fatores importantes que afetam o potencial de formação de biofilme (TANG et al., 2011).

Fatores ambientais tais como pH, temperatura, osmolaridade, níveis de O_2, a composição de nutrientes e a presença de outras bactérias, também

desempenham um papel importante no processo de formação de biofilme (HABIMANA et al., 2010).

A remoção de biofilme na indústria acaba sendo dificultosa, devido a presença de exopolissacarídeo secretado pelos microrganismos e das dificuldades relacionadas com a limpeza de equipamento complexos de processamento (MARCHAND et al., 2012).

Microrganismos presentes no biofilme tem como vantagem a alta concentração de nutrientes, facilidade de trocas genéticas, além de maior capacidade de suportar falta de nutrientes, mudanças de pH e maiores concentrações de antibióticos devido à proteção exercida pelo exopolissacarídeo (PALMER et al., 2007; BURGESS et al., 2010; NGUYEN & YUK, 2013).

Essas vantagens dos biofilmes têm sido evidenciadas quando várias espécies de bactérias estão presentes, pois os biofilmes multiespécies foram observados sendo mais resistentes ao estresse do ambiente (SCHWERING et al., 2013; GIAOURIS et al., 2013). Dessa forma, as bactérias patogênicas podem coexistir dentro desta estrutura com outros organismos ambientais; um exemplo disto é *Listeria monocytogenes*, que sobrevive no biofilme de *Pseudomonas* (MARCHAND et al., 2012).

3.5 Atividade enzimática no Biofilme

A produção de enzimas deteriorantes dentro de biofilmes não tem sido reconhecida pela indústria de processamento de leite como uma fonte de contaminação de produtos lácteos (TEH et al., 2014). No entanto, estudos recentes demostram que a proteólise e a lipólise produzidas por células bacterianas isoladas de laticínios, apresentaram-se superiores dentro do biofilme, enquanto que fora, como células planctônicas a atividade enzimática foi menor (TEH et al., 2012; TEH et al., 2013).

Esses resultados mostram que biofilmes dentro do ambiente de laticínios são uma fonte de enzimas de deterioração que podem ter importantes consequências econômicas para os fabricantes de produtos lácteos (TEH et al., 2014).

A maior atividade enzimática dentro do biofilme pode ser explicada pela diferença de atividades metabólicas e a fisiologia destas estruturas comparado com

células planctônicas (OOSTHUIZEN et al., 2001), pois sabe-se que microrganismos organizados em biofilmes possuem mais vantagens (BURGESS et al., 2010; NGUYEN & YUK, 2013).

Alguns estudos, no qual as atividades enzimáticas foram maiores em biofilmes do que em células planctônicas, observaram que o acúmulo de enzimas dentro do biofilme também pode ajudar na sobrevivência das bactérias dentro de um ambiente de laticínios (FRØLUND et al., 1995; GAMARRA et al., 2010).

As condições em que as bactérias crescem (como biofilme ou como células planctônicas) podem influenciar na interação e produção da enzima entre as bactérias. A expressão genética é diferente em bactérias dentro biofilmes do que na população de células planctônicas (DAVEY & O'TOOLE, 2000; OOSTHUIZEN et al., 2001; SCHEMBRI et al., 2003).

Dessa forma, uma vez que a formação de biofilme e a produção de enzimas extracelulares por bactérias psicrotróficas são responsáveis pela deterioração de produtos na indústria de laticínios, medidas de controle devem ser adotadas para assegurar a qualidade do produto final (TEH et al., 2014).

3.6 Impacto dos Biofilmes na Indústria de Alimentos

Os biofilmes elevam a carga microbiana do leite e de seus derivados durante o processamento e, muitas vezes contamina-os com patógenos. Consequentemente colocam em risco a saúde do consumidor e ocasionam prejuízos financeiros à indústria em decorrência da diminuição da vida de prateleira do leite e derivados lácteos (MIGUEL et al., 2014).

A formação de biofilmes microbianos em superfícies da indústria de lácteos, principalmente em trocadores de calor, é uma preocupação constante (MIGUEL et al., 2014). Os microrganismos em biofilmes catalisam reações químicas e biológicas que causam corrosão do metal em tubulações e tanques e podem reduzir a eficácia de transferência de calor se uma camada de biofilme tornar-se suficientemente espessa nessa superfície (SANTOS et al., 2011a).

Na indústria de lácteos, a formação de biofilmes está condicionada a diferentes variáveis, como design adequado de equipamentos, qualidade da matéria-prima, eficiência do processo de higienização, dentre outros (JOSEPH et al., 2001).

A falta de controle e a limpeza e sanitização inadequadas adotadas para esses equipamentos, com formação de biofilmes, podem definir a extensão de possíveis problemas na qualidade e tempo de prateleira de leite pasteurizado e defeitos em queijos processados a partir de leite com altas contagens desses microrganismos (GÂNDARA, 2000; GUTIÉRREZ et al., 2012).

No setor lácteo, resíduos de leite, especialmente proteínas e sais minerais – como fosfato de cálcio – geralmente são constituintes da matriz de biofilmes em tanques e tubulações (BREMER et al., 2006; SANTOS et al., 2011; LEI et al., 2011). A adesão de bactérias a superfícies de aço inoxidável é um fenômeno recorrente que requer adequada limpeza, desinfecção e vigilância constantes. Após o uso, a limpeza química é sistematicamente aplicada nos equipamentos e superfícies alimentares. No entanto, esta operação, quando não executada devidamente, pode eliminar apenas alguns dos microrganismos aderidos deixando outros na superfície (BOULANGÉ-PETERMANN et al., 2004).

Ainda, de acordo com Souza et al. (2014) e Marino et al. (2011), as condições higiênicas dos manipuladores de alimentos têm sido associadas à dispersão de linhagens de *S. aureus* produtoras de biofilmes dentro da indústria alimentícia.

Como consequência, todos esses fatores podem acarretar problemas graves e perdas econômicas devido à diminuição da vida de prateleira do produto, além de ser considerada uma fonte crônica de recontaminação por patógenos alimentares (MIGUEL et al., 2014).

Levando em consideração que os biofilmes possuem caráter protetor aos microrganismos, tornando, assim, uma fonte resistente de contaminação à ação de agentes químicos e físicos, como sanitizantes empregados nos processos de higienização (SIMÕES et al., 2010; PARQUE et al., 2012), a prática mais eficiente para evitar essa problemática é impedindo a formação de biofilmes, incluindo boas práticas de higiene na produção e o uso efetivo de produtos de limpeza e desinfecção (PARQUE et al., 2012).

4. ARTIGOS CIENTÍFICOS

Neste estudo, serão apresentados três artigos científicos, das seções 4.1 à 4.3.

4.1 Artigo 1: Deterioration potential and biofilm formation by psychrotrophic bacteria isolated from buffalo milk

Deterioration potential and biofilm formation by psychrotrophic bacteria isolated from buffalo milk

Marciele Bogo[1], Karine Lauer Cruz[2], Álvaro Gonzalez Revello[3], Ana Paula Guedes Frazzon[1] and Amanda de Souza da Motta[1]

[1]Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil. [2] Universidade Feevale, Novo Hamburgo, Brazil. [3] Instituto de Ciencia y Tecnología de los Alimentos de Origen Animal – Departamento de Ciencia y Tecnología de la Leche – Facultad de Veterinaria - UDELAR, Montevideo, Uruguay.

*Corresponding author: Instituto de Ciências Básicas da Saúde, Departamento de Microbiologia, Imunologia e Parasitologia, Rua Sarmento Leite 500, sala 216, 90050-170 Porto Alegre, Brazil; e-mail: amanda.motta@ufrgs.br

Abstract

The aim of the study was to investigate the enzyme activity and evaluate the production of biofilms for 21 bacteria isolated from refrigerated buffalo raw milk. The proteolytic, lipolytic and lecithinase activity, and the production of exopolysaccharides were evaluated at different temperatures. The biofilm formation in microtiter plates, was evaluated at different temperatures and in presence of residual milk buffalo and bovine for all psychrotrophic bacteria. All cultures showed proteolytic profile, 9 cultures showed lipase activity and lecithinase production was found in 7 of the evaluated psychotropic bacteria. The ability to produce exopolysaccharide was found in 12 bacteria. Of the 21 bacterial isolates, 16 were biofilm forming to 7 °C. At 23 °C, 20 isolates were biofilm formers. For the temperature of 37 °C, biofilm formation was weak for 17 isolates. In biofilm formation in the presence of residual milk buffalo and bovine, 7 and 16 bacteria were biofilm forming, respectively. The results of this study show that many isolated of psychrotrophic bacteria from buffalo raw milk

have the potential to produce extracellular enzymes as well as biofilm formation, which deserves special attention to the best practices in getting the raw milk and in processing establishments milk.

Key-words: psychrotrophic bacteria, proteolysis, lipolysis, biofilms

1. Introduction

The production of buffalo milk in Brazil began to be recognized from the 1990. This milk has special physical and chemical characteristics compared to cow's milk with higher content of protein, fat and lactose [1]. For these characteristics, the industrialization has generated differentiated products, especially the buffalo mozzarella cheese [2].

The quality of milk depends on its microbiological characteristics, which are directly related to the management processes of the dairy herd and the obtaining and maintenance of this feedstock [3]. Regarding the milk microbiota, it may change by factors such as temperature, handling of the animals, utensils sanitization and storage time [4].

The milk cooling process in the farms immediately after milking, allows the multiplication of mesophilic bacteria, a major cause acidification [5]. This treatment is part of the requirements in the legislation so that they can keep the properties of the feedstock [6]. However, the storage temperature of the milk often varies between 4 °C and 10 °C, which allows for the development of psychrotrophic microorganisms [7].

The production of buffalo milk in the southern region of Brazil is 130 thousand liters of milk per year, corresponding to 8,71% of all production in the country [8]. With this production, buffalo milk sector in this region has a concern with the quality of products made of this raw material, since according to Osman et al. [9], buffalo milk also presents microbial contaminants that may compromise the production of dairy products.

Psychrotrophic bacteria are able to growth at low temperature at around 7 °C, but have its optimal temperature higher growth, 20 °C to 30 °C, and these bacteria are the principal agents of deterioration raw milk refrigerated and dairy products. The spoilage action of psychrotrophic bacteria is mainly due to the production of extracellular enzymes that hydrolyze the main components of milk such as proteins and fats [10].

The action of proteolytic enzymes is associated with the promotion of the bitter taste of milk due to hydrolysis of peptide bonds [11]. As these enzymes are present in milk and its dairy products, in low concentrations, over time these enzymes may alter the physicochemical properties of the products, resulting in changes of properties by itself remain active after milk receiving its heat treatment (change of color and flavor) [11, 12]. Lipases are enzymes that catalyse the hydrolysis of triglycerides (triacylglycerols), the major lipid components of milk. The products of the reactions are non-esterified fatty acids, partial glycerides (mono- and diglycerides) and in some cases, glycerol. The lipolysis of milk fat contributes to off-flavours, such as rancid, soapy or occasionally bitter tastes [13].

Another feature of psychrotrophic bacterias is the ability to survive in liquid adhered to the contact surfaces forming cell aggregates called biofilms. The presence of biofilm in the dairy industry represents a risk to consumer health due to the likelihood of the spread of pathogenic bacteria and their toxins, and can cause financial losses due to the decrease product shelf life [14].

According Abe et al. [15] biofilms are microbial cell aggregates embedded in a polymeric matrix formed by exopolysaccharide (EPS) connected to a biotic or abiotic surface. The bacteria forming the biofilm have the advantage of a higher concentration of nutrients, ease of genetic changes, increased ability to withstand the lack of nutrients to pH changes and to the highest concentrations of antibiotics due to the protection exerted by the EPS [16].

The use of buffalo milk has increased in recent years [17], but the study of these microorganisms in the milk is not well established. The objective of this study was to evaluate the production of proteolytic and lipolytic enzymes and check the biofilm forming ability psychotropic bacteria isolated from refrigerated raw milk buffalo.

2. Materials and Methods

2.1 Bacterial cultures and cultivation conditions

The 21 strains of psychrotropic bacteria were previously isolated (in previous work) from refrigerated buffalo raw milk samples, from cooling tanks located in Cooperative. After selection of the bacterial colonies to be worked each isolated received an initial coding, PL, to be subsequently subjected to different tests of characterization and identification.

The cultures were initially kept frozen at -20°C in 20 % glycerol. For the reactivation of the isolates, was used the medium Tryptone Soy Broth (TSB, Himedia, India) with incubation of isolated in temperature of 30 °C for 48 hours and after spread in Tryptone Soy Agar (TSA, Himedia, India) for 48 hours, to observe the purity of the cultures.

2.2 Identification of bacterial isolates

The identification of the psychrotrophic bacterial isolates was performed with the use of morphological and biochemical tests according MacFaddin [18]. The morphological, cultural and physiological assessments were compared with the data described in Bergey's Manual of Determinative Bacteriology [19]. For biochemical evaluation was also used API 20E kit, according to the manufacturer's instructions (bioMérieux, Marcy l'Etoile, France). The galleries API were examined after 24 and 48 hours at 37 °C using as positive control *Escherichia coli* ATCC 10536. The identification was performed manually by sending the seven-digit code to database on-line apiweb ™.

2.3 Evaluation of proteolytic, lipolytic and lecithinase activity

The verification of the enzymatic properties was performed according Ruaro et al. [20] with some modifications. For proteolytic activity, bacterial cultures were inoculated in milk agar (5 g L^{-1} meat peptone, 3 g L^{-1} yeast extract, 12 g L^{-1} agar, 10 % skim bovine milk) and incubated at 30 °C for 48 hours. *Pseudomonas aeruginosa* ATCC 27853 was used as positive control. For lipolytic activity, the psychrotrophic bacterial isolates were inoculated in culture medium Tributyrin Agar (Sigma, EUA) and incubated at temperature of 30 °C for 48 hours. *Staphylococcus aureus* ATCC 25923 was used as positive control. In both tests, the appearance of clear zones around the colony represented enzymatic activity and the results were expressed in millimeters (mm).

Verification of lecithinase production was carried out according to the methodology proposed by Marques et al. [21] with some modifications. The psychrotrophic bacteria were inoculated on Baird Parker agar (Himedia, India) supplemented with 10 % egg yolk emulsion (Kasvi, Italy). The inoculated plates were incubated at 30 °C for 48 hours. *S. aureus* ATCC 25923 was used as positive control. The appearance of opaque zones around the colonies was indicative of enzyme production. The all tests were performed in duplicate.

2.4 Antimicrobial susceptibility testing

The evaluation of the antibiotic susceptibility was performed by the use of the Kirby-Bauer diffusion disc method and described according to the Clinical and Laboratory Standards Institute (CLSI) [22].

The inoculum from each culture were prepared by suspension of the isolated in saline solution and adjusted to the range of 0.5 McFarland Scale (1,5 x 10^8 CFU/ml). The suspensions were inoculated with sterile cotton swab over the surface of agar plates Mueller

Hinton agar (Oxoid, UK). The following concentrations of antibiotics and discs were used: ampicillin 10 mg, ciprofloxacin 5 mg, chloramphenicol 30 mg, erythromycin 15 mg, nitrofurantoin 300 µg, norfloxacin 10 mg and tetracycline 30 mg. The plates were incubated for 48 hours at 30 °C. For control was used *E. coli* ATCC 25922. The results were interpreted according to the criteria established by the CLSI [22].

2.5 Evaluation of exopolysaccharide production by the Red Congo Agar method

The EPS detection was by the method of Freeman et al. [23], using Congo Red Agar (CRA) prepared according described to this author. Plates of the Red Congo Agar medium were inoculated and incubated at 30 °C for 24 hours and 7 °C for 72 hours. For the controls, were used *Staphylococcus epidermidis* ATCC 35984 (strong EPS-producer) and *Staphylococcus carnosus* P-9-4 isolated morcilla (not EPS-producer). EPS positive strains produced black colored colonies while EPS negative strains were pink colored.

2.6 Evaluation of biofilm formation

For the biofilm formation was employed the method described by Stepanovic et al. [24]. After bacterial growth on TSA plates, the colonies were resuspended in 0,85% saline solution and turbidity was standardized according to the McFarland scale of 0.5, which corresponds to 1,5 x 10^8 CFU/ml.

The assay was performed in 96 well polystyrene microtiter plates (NEST, China). The wells were filled with 180 µl of TSB (Himedia, India), plus 0,25% glucose and 20 µl of bacterial suspension. Each isolate was inoculated in octoplicate. For negative control, wells were inoculated in the same conditions but without the presence of the isolates. For the positive control was used a strong former culture biofilm *S. epidermidis* ATCC 35984. Plates were prepared and three replicates were used for incubations at different conditions: 72 °C for

7 hours, 23 °C for 24 hours and at 37 °C for 24 hours, to evaluate the formation of biofilms. After incubation the wells were aspirated and samples were washed three times with 200 µl of 0,85% saline solution. The fixing of the bacteria was performed using 200 µl of methanol PA for 20 minutes. After the methanol was aspirated and the microplates were inverted, allowing it to dry overnight at room temperature.

The staining procedure was performed with 200 µl of a solution of crystal violet 0,5% for 15 minutes, followed by washing the plate with sterile distilled water. After drying the plate, the bacterial cells fixed and stained at the bottom of the wells were resuspended in 200 µl of 95 % ethanol for 30 minutes and then the quantification of the biofilm was made. The optical density (OD) of the bacterial biofilm was quantified with the aid of a microplate reader spectrophotometer at a wavelength of 450 nm (Anthos 2010 Type 17 550 S. Nº 17 550 4894). Isolates were sorted according Chiari et al. [25] as strong, moderate, weak or no biofilm formers.

2.7 Biofilm formation assay with milk residual

To simulate the presence of residual milk on a surface of sterile microtiter plates, the wells were filled with 200 µl of bovine and buffalo milk (pasteurized whole), before the biofilm formation assay. The incubation was made at 7 °C for 24 hours to simulate a condition of a milk storage tank. After incubation, the entire volume of milk was aspirated from the microtiter plate wells. The biofilm formation capacity of psychrotrophic bacteria in the presence of milk residues was performed by incubating the cultures at 7 °C for 72 hours and 30 °C for 24 hours. The procedure for biofilm formation, reading and interpretation was made according to item 2.6

Results and discussion

3.1 Identification of psychrotrophic bacteria

The psychotropic bacteria were characterized and identified by biochemical tests and using the API 20E kit. According to the tests, the following bacterial genera have been identified: *Pseudomonas, Chryseobacterium, Enterobacter, Burkholderia, Acinetobacter* and *Oligella* (Table 1). Among the Gram-negative bacteria, *Pseudomonas* was a bacterial genus more isolated. We know the importance of this genus because it has species that cause deterioration in milk and milk products [26]. This is because these bacteria are present in the environment and can be transmitted to the raw milk from soil, water, vegetation and in dairy farms [27]. The species belonging to the genera *Chryseobacterium, Burkholderia* and *Acinetobacter* are common food contaminants, and also isolated from milk and have a high potential for food spoilage [28].

3.2 Proteolytic, lipolytic and lecithinase production by psychrotrophic bacteria

In this study, the 21 bacterial isolates showed proteolytic degradation halos on agar milk with values observed between 4 mm and 17 mm (Table 1). This profile demonstrates the ability to hydrolyze casein, the principal milk protein component through the production of proteolytic enzymes. The production capacity of these compounds has been shown by other authors, especially the *Pseudomonas* genus as the main involved with this profile [11, 29, 30]. Technologically these activities have been linked to the loss of cheese yield, the formation of off-flavors, gelation and coagulation of the UHT milk proteins (ultra high temperature) during storage, which limits the milk shelf life and dairy products [29].

For the lipolytic profile, nine (42,85 %) isolates showed lipid degradation halos with values between 5 mm and 8 mm (Table 1), and *Chryseobacterium* sp. PL6.4 showed the

largest halos. The lipase activity is a problem for the dairy industry, because the hydrolysis of lipids present in the layer of fat globules, leading to alteration of the flavor and properties of milk and dairy products [31]. This compromises the quality of dairy products reduces its shelf life. A similar effect occurs when bacteria produce lecithinase enzyme, since this also acts on milk fat globules causing changes in cream, for example [32]. The lecithinase production was found in seven (33 %) isolates (Table 1). With these tests it was observed that four (19 %) bacteria showed the three evaluated enzymatic properties which highlights the negative impact of the presence of these isolated on buffalo milk, considering the negative impact on the final quality of dairy products.

Table 1. Evaluation of enzymatic activity, antimicrobial resistance profile and production of exopolysaccharides by psychotropic bacteria isolated from refrigerated buffalo raw milk.

Strains	PA (mm)	LA (mm)	L	ARP	EPS	
					7 °C	30 °C
B. mallei PL3.2	8	-	-	AMP/NITR	+	+
P. aeruginosa PL3.4	13	5	-	AMP/CLOR/ERIT/NITR	-	-
P. fluorescens PL3.5	10	5	+	AMP/ERIT/NITR	-	-
P. putida PL4.1	17	7	-	AMP/ERIT/NITR	-	+
P. putida PL4.2	8	5	-	AMP/NITR	-	+
Enterobacter sp. PL4.4	6	-	-	AMP/ERIT/NITR	-	-
P. fluorescens PL4.5	10	-	-	AMP/ERIT/NITR	-	-
P. fluorescens PL5.2	4	-	-	-	+	+
P. aeruginosa PL5.4	7	7	+	AMP/NITR	-	+
A. johnsonii PL5.6	8	-	-	AMP/NITR	-	+
P. putida PL6.2	4	-	-	AMP/NITR	-	-
P. putida PL6.3	6	-	-	AMP/NITR	-	-
C. indologenes PL6.4	10	8	-	AMP/NITR	-	+

P. fluorescens PL7.1	7	7	-	AMP/ERIT/NITR	-	-
O. urethralis PL7.2	7	5	-	AMP/CLOR/ERIT/NITR	-	+
Enterobacter sp. PL7.3	9	-	-	-	+	+
A. radioresistens PL7.4	15	-	+	AMP/ERIT/NITR	-	+
C. indologenes PL8.1	11	5	+	AMP/ERIT/NITR	-	+
A. radioresistens PL8.2	14	7	+	AMP/CLOR/ERIT/NITR	-	-
A. radioresistens PL8.3	13	-	+	AMP/ERIT/NITR	-	+
A. radioresistens PL8.5	15	-	+	AMP/ERIT/NITR	-	-
P. aeruginosa ATCC 27853	15	NA	-	NA	NA	NA
S. aureus ATCC 25923	NA	11	+	NA	NA	NA
S. epidermidis ATCC 35984	NA	NA	NA	NA	+	+
S. carnosus ATCC 12228	NA	NA	NA	NA	-	-

PA: Proteolytic Activity; AL: Lipolytic Activity; L: Lecithinase; ARP: Antimicrobial Resistance Profile; EPS: Formation of exopolysaccharideo; AMP (10 mg): Ampicillin (10 mg); CIPR (5 mg): Ciprofloxacin (5 mg); CLOR (30 mg): chloramphenicol (30 mg); ERIT (15 mg): Erythromycin (15 mg); NITR (300 mg): Nitrofurantoin (300 mg); NORFL (10 mg): Norfloxacin+A27:H53 (10 mg); TETR (30 mg): Tetracycline (30 mg); NA: Not applicable.

3.3 Antimicrobial susceptibility tests

According to the interpretation of antimicrobial susceptibility testing it was possible to observe a high percentage of resistance to ampicillin (10 mg) and nitrofurantoin (300 mg) (90,5 %) except for *P. fluorescens* PL5.2 isolates and *Enterobacter* sp. PL7.3 showing sensitivity profile. To erythromycin (15 mg), 13 bacterial isolates were resistant and 6 had intermediate resistance. All isolates were sensitive to ciprofloxacin (5 mg), norfloxacin + A27: H53 (10 mg) and tetracycline (30 mg).

The resistance profile presented by these isolates, in three or more classes of antibiotics, was observed in 57,14% of the isolates analyzed, showing a multidrug resistance profile. Importantly this point because these microorganisms once present in food, they

become reservoir of resistance genes, being possible to exchange genetic material with other bacteria that may be present in the final product to be consumed by humans [33, 34].

3.4 Evaluation of exopolysaccharide production by the red congo agar method

Of the 21 isolates of psychrotrophic bacteria, it was found that three of these cultures were positive for production of this compound at 7 °C and twelve were positive for EPS production at 30 °C. The production of EPS was evidenced by the appearance of colonies black color characteristics and dried crystalline consistency.

The ability to form EPS in congo red agar is indicative that these bacteria have the ability to form biofilms, knowing about the importance of this polysaccharide in the structure of the biofilm. It is known that the congo red can interact directly with certain polysaccharide forming colored complex [35]. However, for the formation of biofilm, bacteria are also dependent on other factors such as the presence of substrate surfaces and determining types of secondary structures for the adhesion process to a certain area [14].

3.5 Biofilm formation

In the biofilm formation evaluations, it was observed that the bacterial isolates showed different behaviors when evaluated at different temperatures, as well as, with the presence of milk residual (Table 2).

Of the 21 psychrotrophic bacteria studied, 16 (76,19%) were considered as forming biofilms 7 °C. Of these 16 isolates, 2 (9,52 %) were classified as moderate forming biofilms and 14 (66,6 %) as poor forming. At 23 °C, 20 isolates (95,23 %) were able to form biofilms. Of these, 3 (14,28 %) were moderate and 17 (80,95 %) weak. For the temperature of 37 °C, 17 (80,95 %) were classified as weak biofilm formers. None of the isolates was determined to

be strong forming biofilm, and the conditions employed. Eleven isolates have been shown to be weak forming biofilms in the three evaluated conditions. The isolated *P. aeruginosa* PL5.4 and *P. fluorescens* PL7.1 presented as weak forming biofilms at 37 °C. At lower temperatures tested, 7 °C and 23 °C, behaved as moderate former.

When the biofilm formation was performed in the presence of residual milk, 4 isolates (19,04 %) were poor formers at 7° C and 17 (80,95 %) did not form biofilms. Three (14,28 %) were moderate formers 23 ° C and 4 did not form biofilm in residual buffalo milk. With the presence of the residual bovine milk, 15 (71,42 %) bacteria were forming weak to 7° C, 1 (4,76 %) was moderate forming and 5 (28,57 %) did not form biofilms. Already at 23 °C, it was observed that only 8 (38,09 %) isolated were weak forming biofilms and 13 (61,9 %) did not form these structures.

Table 2. Biofilm formation in different conditions by psychotropic bacteria isolated from raw milk of buffalo.

Strains	Biofilme sem resíduo			Biofilm residual milk buffalo		Biofilm residual milk cow	
	37°C	23°C	7°C	23°C	7°C	23°C	7°C
B. mallei PL3.2	Weak	Weak	NF	NF	NF	NF	NF
P. aeruginosa PL3.4	Weak	Weak	Weak	NF	NF	NF	Weak
P. fluorescens PL3.5	Weak	Weak	Weak	NF	NF	NF	Weak
P. putida PL4.1	Weak	MD	Weak	NF	NF	NF	NF
P. putida PL4.2	Weak	Weak	NF	NF	NF	Weak	Weak
Enterobacter sp. PL4.4	Weak	Weak	Weak	MD	NF	NF	Weak
P. fluorescens PL4.5	Weak	Weak	Weak	MD	NF	Weak	Weak
P. fluorescens PL5.2	Weak	Weak	NF	MD	NF	NF	Weak
P. aeruginosa PL5.4	Weak	MD	MD	NF	NF	NF	Weak
A. johnsonii PL5.6	Weak	Weak	Weak	NF	NF	NF	NF

P. putida PL6.2	NF	Weak	Weak	NF	Weak	Weak	Weak
P. putida PL6.3	Weak	Weak	Weak	NF	NF	NF	NF
C. indologenes PL6.4	NF	NF	Weak	NF	NF	Weak	Weak
P. fluorescens PL7.1	Weak	MD	MD	NF	Weak	Weak	MD
O. urethralis PL7.2	Weak	Weak	Weak	NF	Weak	NF	NF
Enterobacter sp. PL7.3	Weak	Weak	NF	NF	Weak	Weak	Weak
A. radioresistens PL7.4	Weak	Weak	Weak	NF	NF	Weak	Weak
C. indologenes PL8.1	Weak	Weak	Weak	NF	NF	NF	Weak
A. radioresistens PL8.2	NF	Weak	Weak	NF	NF	NF	Weak
A. radioresistens PL8.3	Weak	Weak	Weak	NF	NF	Weak	Weak
A. radioresistens PL8.5	NF	Weak	Weak	NF	NF	NF	Weak
S. epidermidis ATCC 35984	Strong	Strong	NF	NF	NF	NF	NF

NF: No Forming Biofilm; MD: Moderate Forming Biofilm.

In the evaluations made without residual milk, the isolates of *P. aeruginosa* PL5.4 and *P. fluorescens* PL7.1 were moderate forming at temperatures of 7 °C and 23 °C. Another isolated which showed similar behavior at 23 °C was *P. putida* PL4.1.

These isolates represent a problem for the dairy industry, because according to Bogino et al. [36] bacteria that these structures organize acquire several advantages including protection against predation, desiccation and exposure to antibacterial substances and better acquisition of nutrients released into the cooling processing or storage environment.

The cleaning improperly, it can lead to deposition of organic matter and microorganisms, with the result of possible adherence and biofilm formation by the bacteria [37]. In the evaluation of biofilm formation test in the presence of milk residual, it was observed that some isolates showed moderate training, where in the absence of milk residual were classified as weak formers. These results indicate that variables such as temperature and the presence of residual are important to be controlled to minimize deposition of these structures that compromise the quality and safety of food.

4. Conclusion

The results of this study show that many psychrotrophic bacterial isolated from refrigerated buffalo raw milk have the potential to produce proteolytic, lipolytic and lecithinase enzymes and also adhesion and biofilm formation. Thus, all these combined factors and presented by psychrotrophic bacterial represent a source of contamination and deterioration to food, causing problems for the economy and public health.

5. Acknowledgements

Authors thank the National Council for Scientific and Technological Development (CNPq), the supplier of the samples of buffalo milk and other laboratories that supported this research, Uniquely the Institute of Food Science and Technology - UFRGS.

6. References

1. Ahmad S, Anjum FM, Huma N, Sameen A, Zahoor T, (2013) Composition and physico-chemical characteristics of buffalo milk with particular emphasis on lipids, proteins, minerals, enzymes and vitamins. J Anim Plant Sci 23: 62-74.

2. Araújo KBS, Rangel AHN, Fonseca FCE, Aguiar EM, Simplício AA, Novaes LP, et al, (2012) Influence of the year and calving season on production, composition and mozzarella cheese yield of water buffalo in the State of Rio Grande do Norte, Brazil. Ital J Anim Sci11: 86-91.

3. Alexopoulos A, Tzatzimakis G, Bezirtzoglou E, Plessas S, Stavropoulou E, Sinapis E, et al. (2011) Microbiological quality and related factors of sheep milk produced in farms of NE Greece. Anaerobe 17: 276-279.

4. Millogo V, Svennersten KS, Ouédraogo GA, Agenäs S, (2010) Raw milk hygiene at farms, processing units and local markets in Burkina Faso. Food Control 21: 1070–1074.

5. Perin LM, Moraes PM, Almeida MV, Nero LA, (2012) Intereference of storage temperatures in the development of mesophilic, psychrotrophic, lipolytic and proteolytic microbiota of raw milk. Ciências Agrárias, Londrina 33: 333-342.

6. Sitohy M, Mahgoub S, Osman A, (2011) Controlling psychrotrophic bacteria in raw buffalo milk preserved at 4ºC with esterified legume proteins. LWT - Food Sci Technol 44: 1697-1702.

7. Pothakos V, Samapundo S, Devlieghere F, (2012) Total mesophilic counts underestimate in many cases the contamination levels of psychrotrophic lactic acid bacteria (LAB) in chilled-stored food products at the end of their shelf-life. Food Microbiol 32: 437-443.

8. Damé MCF, De Lima CT, Marcondes CR, Ribeiro MER, Garner ADV, Santos CS, (2010) Production and quality of buffalo milk in Rio Grande do Sul: Preliminary data. Embrapa Clima Temperado. 1-46.

9. Osman AO, Mahgoub SA, Sitohy MZ, (2014) Hindering milk quality storage deterioration by mild thermization combined with methylated chickpea protein. Int Food Res J 21: 693-701.

10. Vithanage NR, Yeager TR, Jadhav SR, Palombo EA, Datta N, (2014) Comparison of identification systems for psychrotrophic bacteria isolated from raw bovine milk. Int J Food Microbiol189: 26-38.

11. Teh KH, Flint S, Palmer J, Lindsay D, Andrewes P, Bremer P, (2011) Thermo-resistant enzyme-producing bacteria isolated from the internal surfaces of raw milk tankers. Int Dairy J 21: 742-747.

12. Button PD, Roginski H, Deeth HC, Craveni HM, (2011) Improved shelf life estimation of UHT milk by prediction of proteolysis. J Food Quality 34: 229–235.

13. Teh KH, Lindsay D, Palmer J, Andrewes P, Bremer P, Flint S, (2013) Lipolysis within single culture and co-culture biofilms of dairy origin. Int J Food Microbiol 163: 129-135.

14. Teh KH, Flint S, Palmer J, Andrewes P, Bremer P, Lindsay D, (2014) Biofilm - An unrecognised source of spoilage enzymes in dairy products? Int Dairy J 34: 32-40.

15. Abee T, Kovacs AT, Kuipers OP, Van Der Veen S, (2011) Biofilm formationand dispersal in Gram-positive bacteria. Curr Opin Biotech 22: 172–179.

16. Palmer J, Flint S, Brooks J, (2007) Bacterial cell attachment, the beginning of a biofilm. J Ind Microbiol Biot 34: 577-588.

17. Han X, Lee FL, Zhang L, Guo MR, (2012) Chemical composition of water buffalo milk and its low-fat symbiotic yogurt development. Funct Food Health Dis 2: 86-106.

18. Macfaddin JF (2000) Biochemical Tests for Identification of Medical Bacteria. Williams & Wilkins. Philadelphia Lippincott.

19. Holt JG (2000) Bergey's manual of determinative bacteriology Baltimore. Lippincott, Williams & Wilkins.

20. Ruaro A, Andrighetto C, Torriani S, Lombardi A, (2013) Biodiversity and characterization of indigenous coagulase-negative *Staphylococci* isolated from raw milk and cheese of North Italy. Food Microbiol 34: 106-111.

21. Marques SC, Evangelista SR, Piccoli RH, (2012) Diversity of psychrotrophic bacteria isolated from the milk cooling collective tanks and antibiotics resistance profile of the microorganisms isolates. Revista Instituto Adolfo Lutz 71: 670-676.

22. Clinical and Laboratory Standards Institute (CLSI) (2014) Performance standards for antimicrobial susceptibility testing: Twenty-first informative supplement. CLSI Document 34: M100-S24.

23. Freeman DJ, Falkiner FR, Keane CT, (1989) New method for detecting slime production by coagulase negative *Staphylococci*. J Clin Pathol 42: 872–874.

24. Stepanović S, Vuković D, Hola V, Di Bonaventura G, Djukić S, Cirković I, et al. (2007) Quantification of biofilm in microtitar plates: overview of testing conditions and practical recommendation for assessment of biofilm production by *Staphylococci*. Acta Path Micro Im B 115: 891-899.

25. Chusri S, Phatthalung PN, Voravuthikunchai SP, (2012) Anti-biofilm activity of Quercus infectoria G. Olivier against methicillin-resistant *Staphylococcus aureus*. Lett Appl Microbiol 54: 511–517

26. Marchand, S., Vandriesche, G., Coorevits, A., Coudijzer, K., de Jonghe, V., Dewettinck, E., et al. (2009). Heterogeneity of heat-resistant proteases from milk *Pseudomonas* species. Int J Food Microbiol 133: 68-77.

27. Vacheyrou M, Normand AC, Guyot P, Cassagne C, Piarroux R, Bouton Y, (2011) Cultivable microbial communities in raw cow milk and potential transfers from stables of sixteen French farms. Int J Food Microbiol 146: 253–262.

28. Giannino ML, Marzotto M, Dellaglio F, Feligini M, (2008) Study of microbial diversity in raw milk and fresh curd used for Fontina cheese production by culture-independent methods. Int J Food Microbiol 130: 188-195.

29. Nornberg MFBL, Friedrich RSC, Weiss RDN, Tondo EC, Brandelli A, (2010) Proteolytic activity among psychrotrophic bacteria isolated from refrigerated raw milk. Int J Dairy Technol 63: 41-46.

30. Baur C, Krewinkel M, Kranz B, Neubeck MV, Wenning M, Scherer S, et al. (2015) Quantification of the proteolytic and lipolytic activity of microorganisms isolated from raw milk. Int Dairy J 49: 23-29.

31. Bekker A, Jooste P, Steyn L, Bothma C, Hugo A, Hugo C, (2016) Lipid breakdown and sensory analysis of milk inoculated with *Chryseobacterium joostei* or *Pseudomonas fluorescens*. Int Dairy J 52: 101-106.

32. Jonghe VD, Coorevits A, Block JÁ, Coillie EV, Grijspeerdt K, Herman L, et al. (2010) Toxinogenic and spoilage potential of aerobic spore-formers isolated from raw milk. Int J Food Microbiol 136: 318–325.

33. Bender EA, Freitas ALP, Reiter KC, Lutz L, Barth AL, (2009) Identification, antimicrobial resistance and genotypic characterization of *Enterococcus* spp. isolated in Porto Alegre, Brazil. Braz J Microbiol 40: 693-700.

34. Frazzon APG, Gama BA, Hermes V, Bierhals CG, Guedes AG, D'Azevedo PA, et al. (2010) Prevalence of antimicrobial resistance and molecular characterization of tetracycline resistance mediated by tet (M) and tet (L) genes in *Enterococcus* spp. isolated from food in Southern Brazil. World J Microb Biot 26: 365-370.

35. Jain A, Agarwal A, (2009) Biofilm production, a marker of pathogenic potential of colonizing and commensal *Staphylococci*. J Microbiol Meth 76: 88-92.

36. Bogino PC, Oliva MM, Sorroche FG, Giordano W, (2013) The role of bacterial biofilms and surface components in plant-bacterial associations. Int J Mol Sci 14: 15838-15859.

37. Miguel EM, De Carvalho AF, De Andrade NJ, Pires ACS, De Paula JCJ, Costa RGB, (2014) Biofilms formation in heat exchangers and its effects on milk and dairy products. Revista Instituto Laticínios Cândido Tostes 69: 53-63.

4.2 Artigo 2: Thermal resistance of proteolytic enzymes produced by psychrotrophic bacteria isolated from refrigerated raw buffalo milk and their effects on milk matrix.

Thermal resistance of proteolytic enzymes produced by psychrotrophic bacteria isolated from refrigerated raw buffalo milk and their effects on milk matrix

Marciele Bógo[1], Karine Lauer Cruz[2], Álvaro Gonzalez Revello[3], Ana Paula Guedes Frazzon[1] e Amanda de Souza da Motta[1]

[1]Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.
[2] Universidade Feevale, Novo Hamburgo, Brasil.

[3] Instituto de Ciencia y Tecnología de los Alimentos de Origen Animal – Departamento de Ciencia y Tecnología de la Leche – Facultad de Veterinaria - UDELAR, Montevideo, Uruguay.

*Corresponding author: Instituto de Ciências Básicas da Saúde, Departamento de Microbiologia, Imunologia e Parasitologia, Rua Sarmento Leite 500, sala 216, 90050-170 Porto Alegre, Brazil; e-mail: amanda.motta@ufrgs.br

Abstract

Given the concern about the impact of the presence of psychrotrophic microorganisms in raw milk, 21 psychotropic bacteria isolated from refrigerated raw buffalo milk were studied. The isolates were tested for evaluation of the proteolytic activity on buffalo milk agar. The cell-free supernatants from the growing of isolates, were obtained for quantification of enzymatic activity at different pH (5.5, 7.0 and 8.0). It was also evaluated the thermal resistance and the clotting ability of proteolytic enzymes in buffalo and bovine milk substrates. All strains were able to produce proteolysis in buffalo milk agar, as well as all cell-free supernatants showed enzymatic activity, with values > 1 U / ml in at least one of pH conditions. Five isolates produced of cell- free supernatant resistant to a heat treatment of pasteurization (63.5 ° C / 30 minutes), being able to coagulate buffalo and bovine milk. The cell-free supernatant obtained from the isolate *P. fluorescens* PL5.4 showed the greater enzymatic activity in a wide pH range (pH 4-10) and an optimum temperature of 40 °C. The cell-free supernatant of this isolate resisted with tested detergents and organic solvents. However, it was not possible to identify the type of protease. The results of this study showed that these microorganisms have the potential to produce proteolytic enzymes, which can compromise the quality of the final product, considering that 100% of the raw buffalo milk, in Rio Grande do Sul/Brazil, is directed to the preparation of dairy products.

K**eywords:** psychrotrophic bacteria/ proteolysis/ thermal resistance/ coagulation

Introduction

Buffalo milk contains higher levels of total solids as crude protein, fat, calcium, phosphorous and slightly higher content of lactose compared with those of cow's milk. However, the consumption of fluid buffalo milk in Brazil is still not widespread, but the high level of total solids makes buffalo milk ideal for processing of dairy products such as cheese (Han et al. 2012). It is particularly used for Mozzarella cheese manufacture and there is no need to concentrate it prior to processing (Sindhu and Arora 2011; Hussain et al. 2012). It is important to consider that milk is an ideal growth medium for many microorganisms, due to its high nutritional value, high water activity and neutral pH (Quigley et al. 2013). The amount of microorganisms present in raw milk is extremely important for the final quality of dairy products, but the presence of unwanted bacteria as spoilage and pathogenic can lead loss of the organoleptic properties of the dairy products until their safety. These microorganisms enter raw milk from a variety of sources, including the teat apex, milking equipment, air, water, feed, grass, soil, environment and operators (Vacheyrou at al. 2011).

The microbiota of raw milk is very diverse and among the contaminating microorganisms, the psychrotrophic bacteria represent an important group because they growth in milk cooling conditions (Quigley et al. 2013). The potential of these microorganisms to thrive in these conditions leads to reduced milk quality, compromising the performance of this product in the production of dairy products (Sitohy et al. 2011). These microorganisms represent from 37% to 54% of the microbiota present in stored refrigerated milk (Raats et al. 2011).

The importance of this microorganism group is due to produce extracellular enzymes such as proteases. Many of these bacterial proteases are heat stable, remaining active over a broad range of temperatures. Heat treatment such as pasteurization and ultra-high temperature (UHT) are not sufficient to inactivate some bacterial proteases (Button et al. 2011).

The enzymatic profile of these bacteria often implicated in the deterioration of dairy products, leads to the deterioration and degradation of caseins, especially k-casein, giving a bitter taste and sour gelling during storage (Åkerstedt et al. 2012; Baglinière et al. 2013).

Proteases can be produced by a variety of bacteria including species of the genus *Bacillus*, *Pseudomonas*, *Burkholderia*, *Chryseobacterium* and *Serratia* found in the dairy environment (Button et al. 2011; Marchand et al. 2012; Vithanage et al. 2014; Bekker et al. 2016).

The proteolytic enzymes are of significant concern for the dairy industry, as it can remain active in milk products during prolonged storage even at low temperatures (Baur et al. 2015). So understanding the importance of the presence of these enzymes in milk is essential to propose reduction strategies of the presence of the producing bacteria.

With increased consumption of products derived from buffalo milk, it is important to study and evaluate the quality of the raw material, because in the state of Rio Grande do Sul - Brazil, 100% buffalo milk are intended for the preparation of dairy products. Thus, this study aimed to evaluate the production of proteolytic enzymes produced by psychrotrophic bacteria isolated from raw buffalo milk and evaluate the possible impact of the presence of these proteases in milk.

Materials and Methods

Bacterial cultures and growing conditions

For this work were studied 21 cultures of psychrotrophic bacteria previously isolated from samples of refrigerated raw buffalo milk and previously identified in another study through biochemical tests and by the API 20E kit (bioMérieux, Marcy l'Etoile, France). These cultures are present in Table 1.

The cultures were initially kept frozen in Tryptone Soy Broth (TSB, Himedia, India) with 20% glycerol at -20 °C. For the reactivation of the isolates, it was used TSB medium with incubation of isolated in temperature of 30 °C for 48 hours. These cultures were inoculated on Tryptone Soy Agar plates (TSA, Himedia, India) for 48 hours to observe the purity of the isolates.

Evaluation of proteolytic activity of psychrotrophic bacteria

Proteolytic activity was evaluated as described by Ruaro et al. (2013) with some modifications. Bacterial cultures were inoculated in buffalo milk agar (5 g L^{-1} meat peptone, 3 g L^{-1} yeast extract, 12 g L^{-1} agar, 10% sterilized buffalo milk) and incubated at 30 °C for 48 hours. *Pseudomonas aeruginosa* ATCC 27853 culture was used as positive control. The proteolytic activity was observed by the appearance of a clear halo around the colony and the results were expressed in millimeters (mm). This test was performed in duplicate.

Evaluation of the thermal stability of proteolytic enzymes

The evaluation of the thermal stability of the proteolytic enzymes was performed according with described by Nornberg et al. (2011) with some modifications. The 21 cultures of psychrotrophic bacteria were grown in 50 ml of sterile milk (5 g powdered skim milk in 50 ml of distilled water) and incubated in a shaking incubator at 180 rpm at 30 °C to 72 hours (Certomat® BS-1). A volume (15 ml) of each culture was centrifuged at 1.900 *x g* for 10 minutes. The obtained fractions, called cell-free supernatant, were filtered through membrane filter of 0.22 µM for the sterilization of aliquots to be evaluated (Millipore, Beford, TX, USA).

A volume of 1 ml of the cell-free supernatant was heat treated at pasteurization temperature (63.5 °C for 30 minutes) in dry bathroom equipment Agrimax and another 1 ml

aliquot remained without heat treatment. The enzymatic quantification was performed according to Daroit et al. (2009).

Milk clotting evaluation

The cell-free supernatant of each of psychrotrophic bacteria (heat treated) was subjected to the milk clotting evaluation. The volume of 1 ml of cell-free supernatant was mixed with 2 ml of pasteurized bovine's milk and the experiment was also conducted with 2 ml pasteurized buffalo's milk. After, it was made the addition of 110 µl of sodium azide 1% (Merck, Darmstadt, Germany) to ensure the inactivation of bacterial cells. The cell-free supernatants without heat treatment were used as control. Evaluation of changes in the inoculated milk, as evidenced by visual observation of coagulation, was observed for 6, 18, 24, 48, 72, 96 and 120 hours with incubation at 7 °C and 30 °C. As a negative control, incubating of milk was made only with the addition of 110 µl of sodium azide 1%, and the positive control was done with commercial chymosin enzyme. This evaluation was performed according to previously described by Nornberg et al. (2011).

Quantification of proteolytic activity

The enzyme production was performed on mineral medium (0.5 g L^{-1} NaCl, 0.3 g L^{-1} KH_2PO_4, 0.4 g L^{-1} K_2HPO_4, 1 g L^{-1} casein) according to Nornberg et al. (2010) and incubation was done at 30 °C, 180 rpm for 72 hours. After incubation, the inoculum (15 ml) was centrifuged at 1.900 *x g* for 10 minutes to give a cell-free supernatant.

The quantification of the proteolytic activity was determined as described by Daroit et al. (2009), using azocasein as substrate. The reaction was initiated by mixing with 100 µl of the cell-free supernatant with 100 µl of azocasein 10 mg / ml and 100 µl buffer (were tested three different pH buffers: Tris-HCl buffer 0.2 M pH 8; saline solution PBS buffer 0.2 M pH

7; and sodium acetate buffer 0.2 M pH 5.5). The mixture was incubated at 37 °C for 30 minutes and the reaction was stopped by addition of 500 µl of trichloroacetic acid (TCA) to 10% (v / v). After centrifugation at 1.900 *x g* for 5 minutes, it was removed 800 µl of supernatant e mixed with 200 µl of NaOH 1.8 M. The absorbance at 420 nm was measured using a spectrophotometer (Biospectro SP-220). Assays were performed in triplicate. One unit of enzyme was defined as the amount of enzyme required for increasing the absorbance at 420 nm of 0.01 under test conditions.

Characterization of the stability of cell-free supernatant

Partial characterization of enzymatic activity was carried out with psychrotrophic bacteria culture that obtained the highest enzymatic activity values in tests done earlier. The methodology was performed according to Mateos et al. (2015) with some modifications. All assays were performed in triplicate.

The cell-free supernatant from a selected culture was used for determination of pH optimum for enzyme activity. Different pH values were tested in the range of 4 to 11 (pH 4 and 5 sodium acetate buffer 0.2 M; pH 6, 7 and 8 sodium phosphate buffer 0.2 M; pH 9, 10 and 11, carbonate bicarbonate buffer 0.2 M) on the cell-free supernatant. To assess the optimal temperature for enzymatic activity were employed at temperatures of 30, 37, 40, 50, 60, 70 and 80 °C in sodium phosphate buffer 0.2 M (pH 7).

The effect of $MgCl_2$ (1 and 5 mM), $CaCl_2$ (1 and 5 mM), NaCl (1 and 5 mM), $CuCl_2$ (1 and 5 mM), $CoCl_2$ (1 and 5 mM), $MnCl_2$ (1 and 5 mM), $ZnSO_4$ (1 and 5 mM), LiCl (1 and 5 mM), $MgSO_4$ (1 and 5 mM), $Al_2(SO_4)_3$ (1 and 5 mM), $FeSO_4$ (1 and 5 mM), sodium sulfate dodecyl (SDS - 1% and 0.5%), Triton X-100 (1% and 0.5%), Tween 20 (1% and 0.5%), dimethylsulfoxide (DMSO - 1% and 0.5%), isopropanol (1% and 0.5%), acetonitrile (1% and 0.5%), ethylenediaminetetraacetic acid (EDTA - 1 mM and 5 mM), fluroride

phenylmethylsulfonylfluoride (PMSF - 1 mM and 5 mM) were evaluated. For these tests, the initial reaction and the proteolytic activity were determined with pre-incubation for 10 minutes at 40 °C with azocasein.

Furthermore, the thermal stability was evaluated by incubating the cell-free supernatant at temperatures of 40, 50, 60, 70, 80, 90 and 100 °C by 10, 20, 30, 45, 60, 90 and 120 minutes. In each assay it was used a negative-enzyme control, replacing the sodium phosphate buffer 0.2 M pH 7.

Results

Evaluation of proteolytic activity of psychrotrophic bacteria

In assessing the proteolytic activity of psychrotrophic bacteria made in buffalo milk agar, it was observed that 21 isolated presented a degradation area around the colony growth, indicating proteolysis. It was possible measure clear halos with measurements ranging from 10 mm to 23 mm. Some isolates produce higher halos when compared to the positive control with halos 15 mm (Table 1).

Coagulation evaluation of bovine and buffalo milk

After follow-up for 5 days of bovine and buffalo milk treated with the cell-free supernatants of 21 psychotropic bacteria cells, coagulation profile was evaluated. It was observed that 5 isolates in different temperatures were capable of promoting total coagulation of both milks at the end of 5 days (Table 2 and Table 3). These data demonstrate the potential that these isolates have to promote coagulation of bovine and buffalo milk. The other psychrotrophic isolates evaluated in the study not produced milk clotting in this study, after heat treatment (63.5 °C for 30 minutes).

The cell-free supernatants of strains *Pseudomonas fluorescens* PL 4.5 coagulated bovine milk initial and *Pseudomonas fluorescens* PL 5.4 coagulated bovine milk partial, in the first 6 hours at a temperature at 7 °C of incubation (Table 2). Furthermore, the cell-free supernatants of strains *Chryseobacterium* sp. PL 6.4 and *P. fluorescens* PL 5.4, in the first 18 hours has presented total coagulation of bovine milk. In the 30 °C incubation, the cell-free supernatant of *P. fluorescens* PL 4.5 and *Chryseobacterium* sp. PL 6.4 isolates showed initial coagulation bovine milk in 6 hours of incubation and after 18 hours with total coagulation (Table 2).

The results of the coagulation profile, for 5 isolates was similar when considered in temperature 7 °C in buffalo milk, where the cell-free supernatants of strains *P. fluorescens* PL 4.5 and *Chryseobacterium* sp. PL 6.4, buffalo milk coagulated in the first 6 hours of observation a temperature at 7 °C (Table 3). However, the total coagulation of buffalo milk of 7 °C was observed in strain *P. putida* PL 4.2 with 48 hours of incubation (Table 3). With the temperature 30 °C it was observed that cell-free supernatants of strains *P. putida* PL 4.2 and *P. fluorescens* PL 5.4 showed initial coagulation and strains *P. aeruginosa* PL 3.4, *P. fluorescens* PL 4.5 and *Chryseobacterium* sp. PL6.4 showed partial coagulation in buffalo milk the first 6 hours of incubation (Table 3). The negative control, incubating of milk was made only with the addition of 110 µl of sodium azide 1%, remained without coagulating the end of the 120 hours of observation, and the positive control was done with commercial chymosin enzyme cogulou 6 hours of incubation.

Evaluation of the thermal stability of proteolytic enzymes

The thermal resistance was determined by enzymatic activity evaluation with azocasein substrate. Were considered thermal resistance cell-free supernatant those produced by 21 psychrotrophics isolates showed activity > 1 Units of proteolysis per ml (U / ml), that

is, the amount of enzyme required for increasing the absorbance at 420 nm of 0.01 under test conditions (Daroit et al. 2009).

The results of stability of cell-free supernatants of heat-treated to 63.5 °C for 30 minute, are shown in Table 1. Following the evaluation of the 21 isolates, it was shown that 5 psychotropic bacteria produced enzyme activity, as noted in the table with values that comprised 7.2 – 11.9, greater than < 1 U / ml.

These bacteria, 4 of them belong to the genus *Pseudomonas* and one of the isolates belong to *Chryseobacterium* genus. The isolated *P. fluorescens* PL 5.4 presented the value 11.9 ± 0.6 U / ml, the most value.

Evaluation of enzyme activity at different pH

Quantification of enzyme activity was performed at three different pH values using azocasein substrate. The optimum pH for the enzyme activity varied among isolates as seen in Table 1. The maximum enzyme activity has been found in isolate of *P. aeruginosa* PL 3.4, with 79 ± 6.3 U / ml at pH 5.5. This even isolated, at pH 7, (value close to the milk pH) also reported to be 57 ± 5.7 U / ml.

In the pH 8, it was found the highest number of strains psychrotrophic with high enzymatic activity and least amount of strains with lower activity than 1 U / ml. Also, 11 strains psychrotrophic showed enzymatic activity in three different pH evaluated.

Characterization and stability of the cell-free supernatant isolate *P. fluorescens* PL 5.4

Partial characterization of cell-free supernatant was performed with isolated *P. fluorescens* PL 5.4, showed the highest enzyme activity values and thermal resistance in the previous tests. In evaluating the effect of temperature it was observed that an increase of temperature to 40 °C was accompanied by increased enzymatic activity, this being considered

the optimum temperature (Fig 1.A). At higher temperatures, this isolate showed enzymatic activity still being observed residual proteolytic activity of 12.9% at 80 °C.

The enzyme activity remains high at pH 4-10, with a maximum loss of 35% of the enzyme activity in pH 10. Maximum activity was observed at pH 7 with sodium phosphate buffer 0.2 M (Fig 1.B). Tests with protease inhibitors were made. However, none of the inhibitors tested in this study (PMSF and EDTA) inactivated the enzyme activity of cell free supernatant of *P. fluorescens* PL 5.4 (Table 4).

The organic solvents were tested to evaluate the maintenance of the stability of enzyme activity with DMSO, acetonitrile and isopropanol, all at concentrations of 1% and 0.5% were made and the results are shown in Fig 2. This shows that the supernatant remained stable under the conditions tested. The effect of different salts was also evaluated. There was a reduction in enzymatic activity when treated with $CuCl_2$ and $FeCl_2$ at 1 mM to 5 mM, with residual activity of 19.56 ± 0.9% and 20.21 ± 0.72%, respectively (Table 5). The enzymatic activity was affected by certain surfactants such as Triton X-100, Tween 80 and SDS (concentration 1 and 0.5%). When SDS was used at concentrations of 0.5 and 1% of residual activity was reduced to 22.79 ± 3.1% and 45.48 ± 2.4%, respectively (Fig 3).

To evaluate the effect of various temperatures over time, aliquots of the supernatants were submitted to different treatments employing 40-100 °C for 0,10, 20, 30, 45, 60, 90 and 120 minutes. It was observed that less than 30% residual activity was maintained with incubations above 60 °C for 10 minutes. Incubation at 40 °C for 10 and 120 minutes resulted in a residual activity of 94% and 49%, respectively. At 50 °C residual activity of 63% and 13% was observed when time periods of 10 and 120 minutes were used, respectively (Fig 4).

Discussion

Psychrotrophic bacteria can produce extracellular proteolytic enzymes that hydrolyze

casein micelles compromising the quality of milk (Baglinière et al. 2013). In our study, the proteolytic profile displayed in buffalo milk agar, demonstrate the potential for hydrolysis of protein constituent of milk.

Considering the milk microbiota, it is known that the presence of psychrotrophic bacteria in high quantity, it is related to the microbiological quality of milk. Among the psychrotropic bacteria, *Pseudomonas* genus is the main cause of deterioration of milk and dairy products, with the microorganisms most frequently isolated from this raw material and the finished product (Marchand et al. 2009). This is because they are ubiquitous in nature and are conveyed to the raw milk from the soil, water and vegetation in dairy farms (Baur et al. 2015).

As our study show and according to other authors, the presence of *Pseudomonas* in raw milk is a possible cause of deterioration of raw milk under refrigerated conditions (Raats et al. 2011; Baglinière et al. 2013; Baur et al. 2015). The study of Baur et al. (2015) confirmed a high prevalence (80%) of enzymatically active Gram-negative isolates, particularly various species of *Pseudomonas*, including *P. aeruginosa* and *P. fluorescens*. In the study of Machado et al. (2015) 26.1% of psychrotropic bacteria found were of the species *P. fluorescens* and *P. lundensis*. The direct correlation between the presence of these microorganisms in milk and milk products and high proteolytic activity presented promotes a reduction in the shelf life of dairy products.

In this study, all isolates showed proteolytic activity when tested on azocasein substrate, confirming the potential of these psychrotropic bacteria with proteolytic potential of the raw buffalo milk. Furthermore, it was observed that the maximal enzyme activity was demonstrated by strain *P. aeruginosa* PL 3.4 at pH 5.5. Considering that the pH of buffalo milk varies between 6.4 and 6.9 (São Paulo 1994; Han et al. 2007; Ménard et al. 2010; Ahmad et al. 2013; De Lima et al. 2014) and bovine milk from 6.6 and 6.8 (Brazil 2011;

Ménard er al. 2010), the proteolytic activity displayed by this psychrotrophic strain was in pH acid found in milk.

An important aspect of proteolytic enzymes is that they are not eliminated by the thermal treatment conventionally used in foods. Thus, once produced by psychrotrophic bacteria in milk, this raw material remain even after treatments such as pasteurization, for example (Button et al. 2011). Five (23.8%) isolates of psychrotropic bacteria tested in this study presented themselves as producers of heat-resistant proteolytic enzymes. In tests, it was evaluated the clotting ability of supernatants coagulate the bovine or buffalo milk, suggesting the risk that these bacteria and their enzymes represent, once present in milk and milk products samples may lead to loss of income and properties of feed. Similar results were found in the study by Teh et al. (2011), 153 isolates capable of producing enzymes, 52 (33.98%) produced heat stable enzymes.

As studied by Braun and Sutherland (2005), the thermal resistance presented by these enzymes occurs by reorganization ability of the tertiary structure of the protein, damaged during pasteurization, making them active, thus being able to deteriorate food. When the clotting test was performed in milk, it was possible to visualize the effect of proteolytic enzyme produced by isolated and their resistance to thermal treatment performed (63.5 °C for 30 minutes). Once the thermal resistant enzymes remain in the milk, is the impairment of the structure of proteins affecting the quality of dairy products as proteases degrade casein micelles, releasing soluble components such as polypeptides and amino acids. These components are lost in the whey and thereby reduce the yield of dairy products and change the standards of identity and quality of food (Baglinière et al. 2013).

According with Chen et al. (2003), the mechanisms involved in the coagulation phenomenon promoted by proteolytic enzymes comprise basically changing milk proteins, the association and dissociation of ions of calcium and dissociation complex k-casein. The

majority of those proteases have the ability to hydrolyze K-casein, β-casein and destabilize the casein micelle, which in turn causes coagulation of the milk. In raw milk K-casein and β-casein are the protein components most susceptible to proteolysis by microorganisms psychrotrophic.

Detection and control protease activity prior to processing are critical because the heat-resistant and able enzyme remain active in foods with different pH and temperature (Zhang and Lv 2014). In addition, knowing the heat resistance characteristics, pH and other variables are important for understanding the behavior of enzymes potentially present in milk samples.

In our study, after we get the results of proteolytic activity tests, it was possible to select a psychrotrophic strain that obtained the highest amount of enzyme activity in the tests. Thus, it was performed a partial characterization of the enzymatic activity of the strain *P. fluorescens* PL 5.4.

The cell-free supernatant of *P. fluorescens* PL5.4 was active at the highest tested temperature (80 °C) with 12.9 ± 1.59% residual activity, meaning that in a process of rapid pasteurization where the milk is processed at 72-75 °C for 15-20 seconds, the enzyme remain active. However, the peak of enzyme activity was observed at the temperature of 40 °C, which is similar to some studies. The proteolytic activity of *Pseudomonas* isolates has been observed to be 40-45 °C (Dufour et al. 2008; Raj et al. 2012; Mateos et al. 2015). Furthermore, it is known that the temperature is a critical variable in some enzymatic processes, while producing the opposite effect to increase the reactivity and enzyme inactivation (Illanes et al., 2000).

In our study, when the binomial reported time / temperature over the cell-free supernatant produced *P. fluorescens* PL5.4 can be observed residual activity of 26.5 ± 2.43% at 70 °C after 30 minutes of treatment, and 12.9 ± 2.1% at 80 °C after 10 minutes of

treatment. Thus, considering the thermal pasteurization treatments used in the dairy industry (pasteurization 62-65 °C for 30 minutes and rapid pasteurization 72-75 °C for 15 to 20 seconds) studied, the supernatant resists these processes, thus representing a possible cause of deterioration of dairy products.

The measured pH conditions demonstrate the maintenance of enzymatic activity below pH 7. Whereas the pH of the milk is between 6 and 7, we observed that the residual activities were 80 ± 2.97% at pH 6 and 100 ± 2.3% at pH 7. Similar results were found in the study of Zambare et al. (2011), and Raj et al. (2012) where the great enzymatic activity to *P. aeruginosa* MCM B-327 was also at pH 7.

The cell free supernatant of *P. fluorescens* PL5.4 has enzymatic activity over a wide pH range (pH 4-10) with optimum pH of 7 and an optimum temperature of 40 °C. The study Zhang and Lv (2014), also found similar results, with activity a range pH 5-10 and optimum pH of 7 on the enzymatic activity of *P. fluorescens* BJ-10.

Sometimes, metal ions are important for enzyme actions and their structural modifications (Zambare et al. 2011). $CuCl_2$, $ZnSO_4$ and $CoCl_2$ in our study has a negligible effect on enzymatic activity *P. fluorescens* PL5.4. Still in the study Zhang and Lv (2014), ions Cu^{2+} had negative effect on the enzymatic activity of *P. fluorescens* BJ-10. Some studies show the enzyme activity increased with the ions Ca^{2+} and Zn^{2+} ((Dufour et al. 2008; Mu et al. 2009). However, in our study these results have not been demonstrated by cell free supernatant of *P. fluorescens* PL5.4. The protease activity resists various detergents and organic solvents.

With the inhibitors used in this study it was not possible to infer more information about the enzymatic activity studied. Other studies are necessary seeking a purification of the enzyme to dive deeper study and understanding of enzyme properties.

The results show that the bacteria isolated from refrigerated raw buffalo milk have the ability to produce extracellular proteolytic enzymes. Some of these isolates producing extracellular proteases with thermal resistance promoting a destabilization of pasteurized milk by hydrolysis of casein.

Because of these characteristics demonstrated by psychotropic bacteria studied, we found that the identification of the presence of the microbiota in raw buffalo milk becomes quite important because it is a raw material understudied. Another point to note is that 100% of the commercialized of buffalo milk in the state of Rio Grande do Sul - Brazil, is aimed at elaboration of dairy products. Thus, lack of skilled labor as well as the non-adoption of best procurement practices in raw material, can compromise the quality of milk and its dairy products.

Acknowledgements

Authors thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and supplier of dairy milk samples and the other laboratories support the Universidade Federal do Rio Grande do Sul.

References

Ahmad S, Anjum FM, Huma N, Sameen A, Zahoor T (2013) Composition and physico-chemical characteristics of buffalo milk with particular emphasis on lipids, proteins, minerals, enzymes and vitamins. J Anim Plant Sci 23: 62-74

Åkerstedt M, Wredle E, Lam V, Johansson M (2012) Protein degradation in bovine milk caused by *Streptococcus agalactiae.* J Dairy Res 79: 297-303

Baglinière F, Matéos A, Tanguy G, Jardin J, Briard-Bion V, Rousseau F, Robert B, Beaucher E, Gaillard JL, Amiel C, Humbert G, Dary A, Gaucheron F (2013) Proteolysis of ultra high temperature-treated casein micelles by AprX enzyme from *Pseudomonas fluorescens* F induces their destabilization. Int Dairy J 31: 55-61

Baur C, Krewinkel M, Kranz B, Neubeck MV, Wenning M, Scherer S, Stoeckel M, Hinrichs J, Stressler T, Fischer L (2015) Quantification of the proteolytic and lipolytic activity of microorganisms isolated from raw milk. Int Dairy J 49: 23-29

Bekker A, Jooste P, Steyn L, Bothma C, Hugo A, Hugo C (2016) Lipid breakdown and sensory analysis of milk inoculated with *Chryseobacterium joostei* or *Pseudomonas fluorescens*. Int Dairy J 52: 101-106

Braun P, Sutherland JP (2005) Predictive modeling of growth and measurement of enzymatic synthesis and activity by a cocktail of selected Enterobacteriaceae and *Aeromonas hydrophila*. Int J Food Microbiol 105: 257-266

Button PD, Roginski H, Deeth HC, Craven HM (2011) Improved shelf life estimation of UHT milk by prediction of proteolysis. J Food Quality 34: 229–235

Chen L, Daniel RM, Coolbear T (2003) Detection and impact of protease and lipase activities in Milk and milkpowders. Int Dairy J 13: 255-275

Daroit DJ, Corrêa APF, Brandelli A (2009) Keratinolytic potential of a novel *Bacillus* sp. P45 isolated from the Amazon basin fish *Piaractus mesopotamicus*. Int Biodeter Biodegr 63: 358–363

De Lima TCC, Rangel AHN, Macêdo CS, De Araújo TPM, De Araújo VM, De Lima Júnior DM, Murmann L, Novaes LP (2014) Composição e qualidade do leite e do soro do leite de búfalas no estado do Rio Grande do Norte. Acta Veterinaria Brasilica 8: 25-30.

Dufour D, Nicodème M, Perrin C, Driou A, Brusseaux E, Humbert G, Gaillard JL, Dary A (2008) Molecular typing of industrial strains of *Pseudomonas* spp. isolated from milk and genetical and biochemical characterization of an extracellular protease by one of them. Int J Food Microbiol 125:188-196

Han BZ, Meng Y, Li M, Yang YX, Ren FZ, Zeng QK, Nout MJR (2007) A survey on the microbiological and chemical composition of buffalo milk in China. Food Control 18: 742–746

Han X, Lee FL, Zhang L, Guo MR (2012) Chemical composition of water buffalo milk and its low-fat symbiotic yogurt development. Functional foods in Health and Disease 2: 86-106

Hussain I, Yan J, Grandison AS, Bell AE (2012) Effects of gelation temperature on Mozzarella-type curd made from buffalo and cows' milk: 2. Curd yield, overall quality and casein fractions. Food Chem 135: 1404–1410

Illanes A, Wilson L, Tomasello G (2000) Temperature optimization for reactor operation with

chitin-immobilized lactase under modulated inactivation. Enzyme Microb Tech 27: 270–278

Kalaiarasi K, Sunitha PU (2009) Optimization of alkaline protease production from *Pseudomonas fluorescens* isolated from meat waste contaminated soil. African J Biotechnol 8: 7035-7041

Machado SG, Da Silva FL, Bazzolli DMS, Heyndrickx M, Costa PMA, Vanetti MCD (2015) *Pseudomonas* spp. and *Serratia liquefaciens* as predominant spoilers in cold raw milk. J Food Sci 80: 1842-1849

Marchand S, Vandriesche G, Coorevits A, Coudijzer K, De Jonghe V, Dewettinck E, De Vos P, Devreese B, Heyndrickx M, De Block J (2009) Heterogeneity of heat-resistant proteases from milk *Pseudomonas* species. Int J Food Microbiol 133: 68-77

Marchand S, De Block J, De Jonghe V, Coorevits A, Heyndrickx M, Herman L (2012) Biofilm formation in milk production and processing environments; influence on milk quality and safety. Compr Rev Food Sci F 11: 133-147

Mateos A, Guyard-Nicodème M, Baglinière F, Jardin J, Gaucheron F, Dary A, Humbert G, Gaillard JL (2015) Proteolysis of milk proteins by AprX, an extracellular protease identified in *Pseudomonas* LBSA1 isolated from bulk raw milk, and implications for the stability of UHT milk. . Int Dairy J 49: 78-88

Ménard O, Ahmad S, Rousseau F, Briard-Bion V, Gaucheron F, Lopez C (2010) Buffalo vs. cow milk fat globules: Size distribution, zeta-potential, compositions in total fatty acids and in

polar lipids from the milk fat globule membrane. Food Chem 120: 544–551

Mu ZS, Du M, Bai Y (2009) Purification and properties of a heat-stable enzyme of *Pseudomonas fluorescens* Rm12 from raw milk. Eur Food Res Technol 228: 725–734

Nornberg MFBL, Friedrich RSC, Weiss RDN, Tondo EC, Brandelli A (2010) Proteolytic activity among psychrotrophic bacteria isolated from refrigerated raw milk. Int J Dairy Tech 63: 41-46

Nornberg MFBL, Mentges ML, Silveira ST, Tondo EC, Brandelli A (2011) A Psychrotrophic *Burkholderia cepacia* strain isolated from refrigerated raw milk showing proteolytic activity and adhesion to stainless steel. J Dairy Res 78: 257–262

Quigley L, O'Sullivan O, Stanton C, Beresford TP, Ross RP, Fitzgerald GF, Cotter PD (2013) The complex microbiota of raw milk. FEMS Microbiol Rev 37: 664-698

Raats D, Offek M, Minz D, Halpern M (2011) Molecular analysis of bacterial communities in raw cow milk and the impact of refrigeration on its structure and dynamics. Food Microbiol 28: 465-471

Raj A, Khess N, Pujari N, Bhattacharya S, Das A, Rajan SS (2012) Enhancement of protease production by *Pseudomonas aeruginosa* isolated from daury effluent sludge and determination of its fibrinolytic potential. Asian Pac J Trop Biomed 5: 1845-1851

Ruaro A, Andrighetto C, Torriani S, Lombardi A (2013) Biodiversity and characterization of

indigenous coagulase-negative *Staphylococci* isolated from raw milk and cheese of North Italy. Food Microbiol 34: 106-111

SÃO PAULO (1194) Secretaria de Agricultura e Abastecimento. Resolução sAA nº 24 de 01 de agosto de 1994: Dispõe sobre as normas técnicas de produção e classificação dos produtos de origem animal e as relativas às atividades de fiscalização e inspeção dos produtos de origem animal. http://cda.sp.gov.br/legislação Accessed 02 september 2015.

Sindhu JS, Arora S (2011) Buffalo milk. Encyclopaedia of Dairy Sciences 2: 503–511

Sitohy M, Mahgou S, Osman A (2011) Controlling psychrotrophic bacteria in raw buffalo milk preserved at 4 °C with esterified legume proteins. LWT - Food Sci Technol 44: 1697-1702

Teh KH, Flint S, Palmer J, Lindsay D, Andrewes P, Bremer P (2011) Thermo-resistant enzyme-producing bacteria isolated from the internal surfaces of raw milk tankers. Int Dairy J 21: 742-747

Vacheyrou M, Normand AC, Guyot P, Cassagne C, Piarroux P, Bouton Y (2011) Cultivable microbial communities in raw cow milk and potential transfers from stables of sixteen French farms. Int J Food Microbiol 146: 253-262

Vithanage NR, Yeager TR, Jadhav SR, Palombo EA, Datta N (2014) Comparison of identification systems for psychrotrophic bacteria isolated from raw bovine milk. Int J Food Microbiol 189: 26-38

Zhang S, Lv J (2014) Purification and properties of heat-stable extracellular protease from *Pseudomonads fluorescens* BJ-10. Food Sci Technol 51: 1185-1190

Zambare V, Nilegaonkar S, Kanekar P (2011) A novel extracellular protease frim *Pseudomonas aeruginosa* MCM B-327: enzyme production and its partial characterization. New Biotechnol 28: 173-181

Table 1. Proteolysis evaluation in buffalo milk agar and enzymatic activity of psychrotropic bacteria isolated from refrigerated raw buffalo milk.

Strains	AP (mm)	Thermal resistance Enzymatic activity (U/ml)	Enzymatic activity (U/ml)		
			pH 5,5	pH 7,0	pH 8,0
Burkholderia mallei PL 3.2	10±	<1	3.9 ± 0.7	5.2 ± 1	5.6 ± 0.9
Pseudomonas aeruginosa PL 3.4	18±	9.5 ± 0.3	79 ± 6.3	57 ± 5.7	73 ± 2.5
Pseudomonas fluorescens PL 3.5	20±	<1	1.6 ± 0.3	7.7 ± 2.3	9.9 ± 3.8
Pseudomonas putida PL 4.1	16±	<1	57.4 ± 0.7	44 ± 2.9	61 ± 4.2
Pseudomonas putida PL 4.2	17±	8.9 ± 0.1	12.9 ± 1.4	33 ± 0.2	37 ± 2
Enterobacter sp. PL 4.4	19±	<1	1.7 ± 0.5	<1	4.7 ± 0.3
Pseudomonas fluorescens PL 4.5	15±	7.2 ± 0.6	<1	11 ± 0.5	3.1± 0.4
Pseudomonas fluorescens PL 5.2	10±	<1	<1	<1	1.5 ± 0.1
Pseudomonas fluorescens PL 5.4	23±	11.9 ± 0.5	32.1 ± 1.3	41 ± 3.4	34 ± 3
Acinetobacter johnsonii PL 5.6	19±	<1	2.9 ± 0.3	<1	<1
Pseudomonas putida PL 6.2	12±	<1	4.7 ± 2.1	1.9 ± 0.5	1.6 ± 1
Pseudomonas putida PL 6.3	15±	<1	4.4 ± 3.9	1.6 ± 0.5	<1
Chryseobacterium indologenes PL 6.4	19±	11.3 ± 0.3	25.9 ± 0.9	18.4 ± 0.5	<1
Pseudomonas fluorescens PL 7.1	17±	<1	9.2 ± 0.6	9.2 ± 1	11.2 ± 0.5

Oligella urethralis PL 7.2	21±	<1	<1	<1	1.9 ± 0.3
Enterobacter sp. PL 7.3	12±	<1	<1	<1	2 ± 0.6
Acinetobacter radioresistens PL 7.4	22±	<1	40 ± 1.1	<1	50 ± 5.7
Chryseobacterium indologenes PL 8.1	20±	<1	23 ± 0.8	23 ± 1	37 ± 2
Acinetobacter radioresistens PL 8.2	20±	<1	37 ± 0.9	34 ± 0.2	37 ± 2
Acinetobacter radioresistens PL 8.3	21±	<1	44 ± 3.8	37 ± 1.1	38 ± 1.1
Acinetobacter radioresistens PL 8.5	22±	<1	40 ± 4	43 ± 0.9	37 ± 1.9
P. aeruginosa ATCC 27853	15±	NAP	NAP	NAP	NAP

NAP: not applicable; AP: Proteolytic Activity.

Table 2. Evaluation of clotting bovine milk by cell-free supernatant of psychrotrophic bacteria isolated from refrigerated raw buffalo milk showed coagulation at 7 °C and 30 °C by 120 hours.

Strains	TT	Observation Time (hours)													
		6	18	24	48	72	96	120	6	18	24	48	72	96	120
		7 °C							30 °C						
P. aeruginosa PL3.4	CFS	- - -	- - -	+ - -	+ - -	+++	+++	+++	- - -	- - -	+ - -	+ - -	+++	+++	+++
	CFSH	- - -	- - -	+ - -	+ - -	++-	+++	+++	- - -	- - -	+ - -	+ - -	+++	+++	+++
P. putida PL4.2	CFS	- - -	- - -	- - -	- - -	+ - -	+++	+++	- - -	- - -	- - -	+ - -	++ -	+++	+++
	CFSH	- - -	- - -	- - -	- - -	- - -	+ - -	++ -	- - -	- - -	- - -	+ - -	++ -	+++	+++
P. fluorescens PL4.5	CFS	+ - -	+++	+++	+++	+++	+++	+++	+ - -	+++	+++	+++	+++	+++	+++
	CFSH	+ - -	+++	+++	+++	+++	+++	+++	+ - -	+++	+++	+++	+++	+++	+++
P. fluorescens PL5.4	CFS	++ -	++ -	++ -	++ -	++ -	++ -	++ -	- - -	+ - -	+++	+++	+++	+++	+++
	CFSH	++ -	++ -	++ -	++ -	++ -	++ -	++ -	- - -	- - -	+ - -	+ - -	++ -	++ -	+++
Chryseobacteriums sp. PL6.4	CFS	+ - -	+++	+++	+++	+++	+++	+++	++ -	+++	+++	+++	+++	+++	+++
	CFSH	- - -	+++	+++	+++	+++	+++	+++	+ - -	+++	+++	+++	+++	+++	+++
Positive control*		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Negative control**		- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -

TT: Treatment; CFS: Cell-Free Supernatant; CFST: Cell-Free Supernatant Heat-treated; - - -; no coagulation; + - -; initiation of coagulation; + + -; partial coagulation; + + +; total coagulation.

* Positive control: only milk (2 mL) with 110 µl of sodium azide 1%.

** Negative control: milk (2 mL) with commercial chymosin enzyme (1 mL).

Table 3. Evaluation of clotting buffalo milk by cell-free supernatant of psychrotrophic bacteria isolated from refrigerated raw buffalo milk showed coagulation at 7 °C and 30 °C by 120 hours.

Strains	TT	Observation Time (hours)													
		6	18	24	48	72	96	120	6	18	24	48	72	96	120
		7 °C							30 °C						
P. aeruginosa PL3.4	CFS	-	-	-	+	-	-	+	++	-	+++	+++	+++	++	-	+++	+++	+++	+++	+++	+++
	CFSH	-	-	-	+	-	-	+	-	-	++	-	+++	+++	++	-	+++	+++	+++	+++	+++	+++
P. putida PL4.2	CFS	-	-	-	+	-	-	++	-	++	-	+++	+++	+++	+	-	-	+++	+++	+++	+++	+++	+++
	CFSH	-	-	-	+	-	-	+	-	-	++	-	++	-	+++	+	-	-	+++	+++	+++	+++	+++	+++
P. fluorescens PL4.5	CFS	+	-	-	+	-	-	++	-	+++	+++	+++	+++	++	-	+++	+++	+++	+++	+++	+++
	CFSH	+	-	-	+	-	-	++	-	+++	+++	+++	+++	++	-	+++	+++	+++	+++	+++	+++
P. fluorescens PL5.4	CFS	-	-	-	+	-	-	++	-	++	-	++	-	+++	+	-	-	+++	+++	+++	+++	+++	+++
	CFSH	-	-	-	+	-	-	+	-	-	++	-	++	-	+++	+	-	-	+++	+++	+++	+++	+++	+++
Chryseobacteriums sp. PL6.4	CFS	+	-	-	++	-	++	-	+++	+++	+++	+++	++	-	+++	+++	+++	+++	+++	+++	+++
	CFSH	+	-	-	+	-	-	++	-	+++	+++	+++	+++	++	-	+++	+++	+++	+++	+++	+++	+++
Positive control*		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Negative control**		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

TT: Treatment; CFS: Cell-Free Supernatant; CFST: Cell-Free Supernatant heat-treated; - - -; no coagulation; + - -; initiation of coagulation; + + -; partial coagulation; + + +; total coagulation.

* Positive control: only milk (2 mL) with 110 µl of sodium azide 1%.

** Negative control: milk (2 mL) with commercial chymosin enzyme (1 mL).

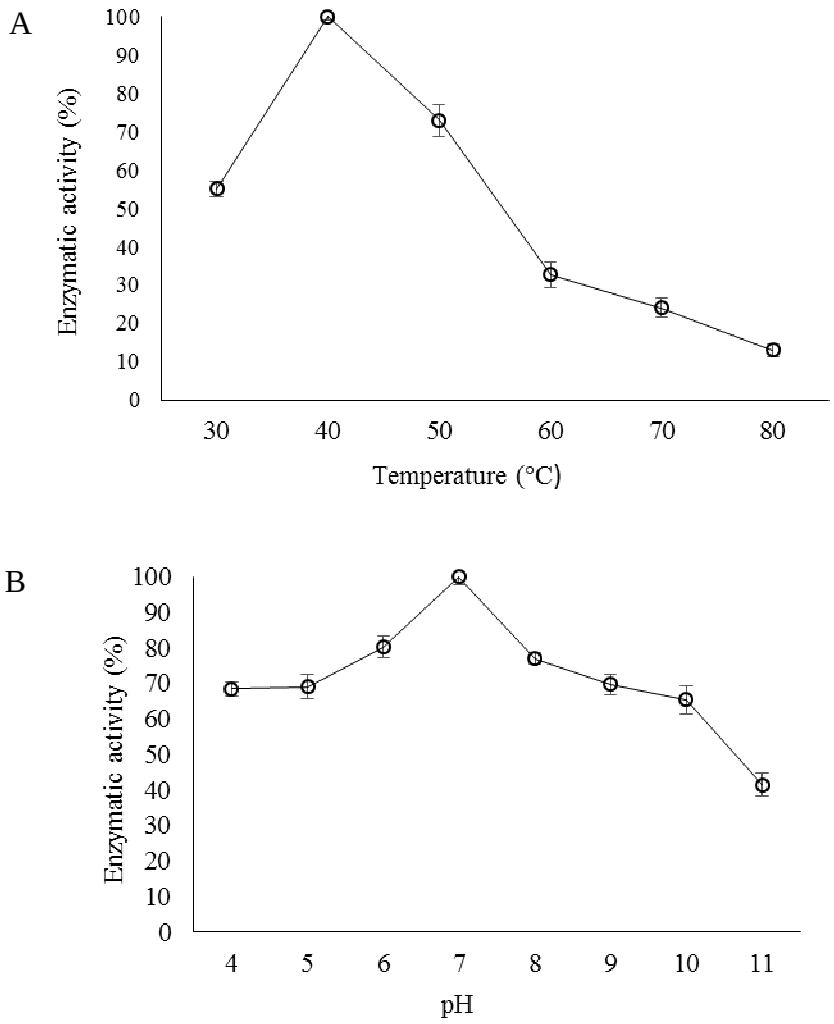

Fig 1. A: Effect of incubation temperature on the proteolytic activity of the cell-free supernatant of *P. fluorescens* PL5.4 using substrate azocasein, with phosphate sodium buffer 0.2 M pH 7. B: Effect of pH on the proteolytic activity of the cell-free supernatant of *P. fluorescens* PL5.4 using substrate azocasein at 40 °C. Results are expressed as % of activity enzymatic. Dates are means of triplicates.

Table 4. Inhibitor effects on the cell-free supernatant produced by the isolated *P. fluorescens* PL5.4, in pre-incubation for 10 minutes, with phosphate sodium buffer 0.2 M pH 7, at 40 °C using substrate azocasein. Results are expressed as % of inhibition of enzymatic activity. Dates are means of triplicates.

Inhibitor	Concentration	Inhibition (%)
PMSF	1 mM	3.45 ± 0.33
	5 mM	4.94 ± 0.2
EDTA	1 mM	22.16 ± 0.43
	5 mM	34.63 ± 0.98

PMSF, Phenylmethylsulphonylfluoride; EDTA, ethylendiaminetetracetic acid disodium salt.

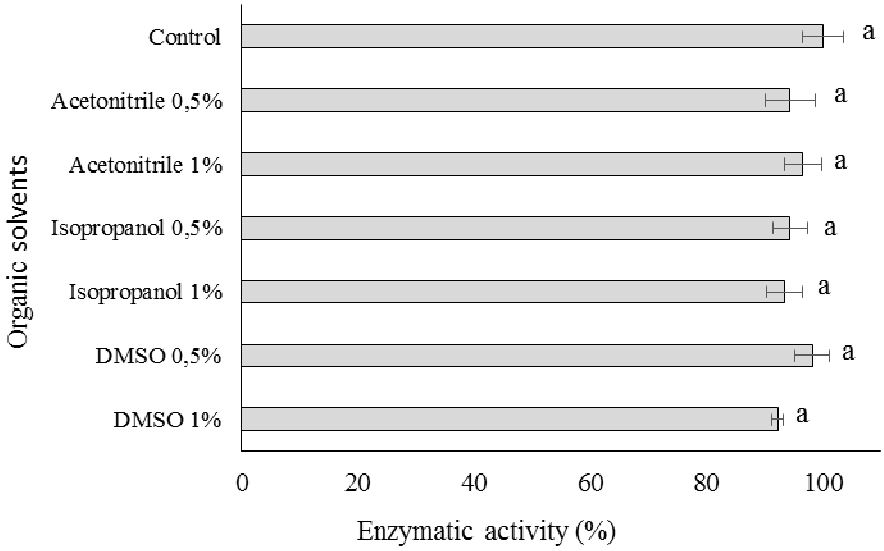

Fig 2. Effects of organic solvents on the cell-free supernatant isolated psychrotrophic *P. fluorescens* PL5.4, in pre-incubation for 10 minutes, with phosphate sodium buffer 0.2 M pH 7, at 40 °C using substrate azocasein. Results are expressed as % of activity enzymatic. Dates are means of triplicates.

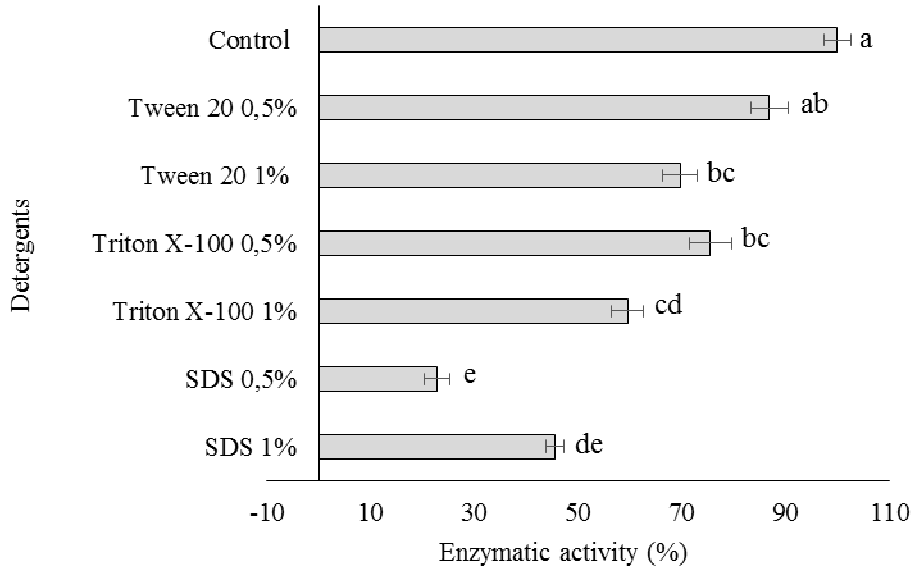

Fig 3. Effects of detergents on the cell-free supernatant isolated psychrotrophic *P. fluorescens* PL5.4, in pre-incubation for 10 minutes, with phosphate sodium buffer 0.2 M pH 7, at 40 °C using substrate azocasein. Results are expressed as % of activity enzymatic. Dates are means of triplicates.

Table 5. Effect of salts of the cell-free supernatant of the strain psychrotrophic *P. fluorescens* PL5.4, in pre-incubation for 10 minutes, with phosphate sodium buffer 0.2 M pH 7, at 40 °C using substrate azocasein. Results are expressed as % of activity enzymatic. Dates are means of triplicates.

Salts	Concentration	Enzymatic activity (%)
Control	-	100 [a]
$MgCl_2$	1mM	73.76 ± 2.1 [bcd]
	5mM	66.02 ± 0.5 [cde]
$CaCl_2$	1mM	63.44 ± 1.9 [def]
	5mM	71.61 ± 2.01 [bcd]
NaCl	1mM	73.33 ± 3.42 [bcd]
	5mM	75.91 ± 0.98 [bcd]
$CuCl_2$	1mM	19.56 ± 0.9 [i]
	5mM	20.21 ± 0.72 [i]
$CoCl_2$	1mM	28.60 ± 1.03 [hi]
	5mM	46.23 ± 1.33 [fg]
$ZnSO_4$	1mM	50.75 ± 2.95 [efg]
	5mM	44.94 ± 4.09 [gh]
$FeSO_4$	1mM	62.77 ± 3.21 [def]
	5mM	72.25 ± 1.83 [bcd]
LiCl	1mM	75.48 ± 2.87 [bcd]
	5mM	70.96 ± 4.54 [bcd]
$MgSO_4$	1mM	81.72 ± 3.22 [bc]
	5mM	77.63 ± 0.98 [bcd]
$Al_2(SO_4)_3$	1mM	84.94 ± 1.87 [ab]

	5mM	84.73 ± 2.36 [ab]
$MnCl_2$	1mM	88.81 ± 2.43 [ab]
	5mM	85.80 ± 5.24 [ab]

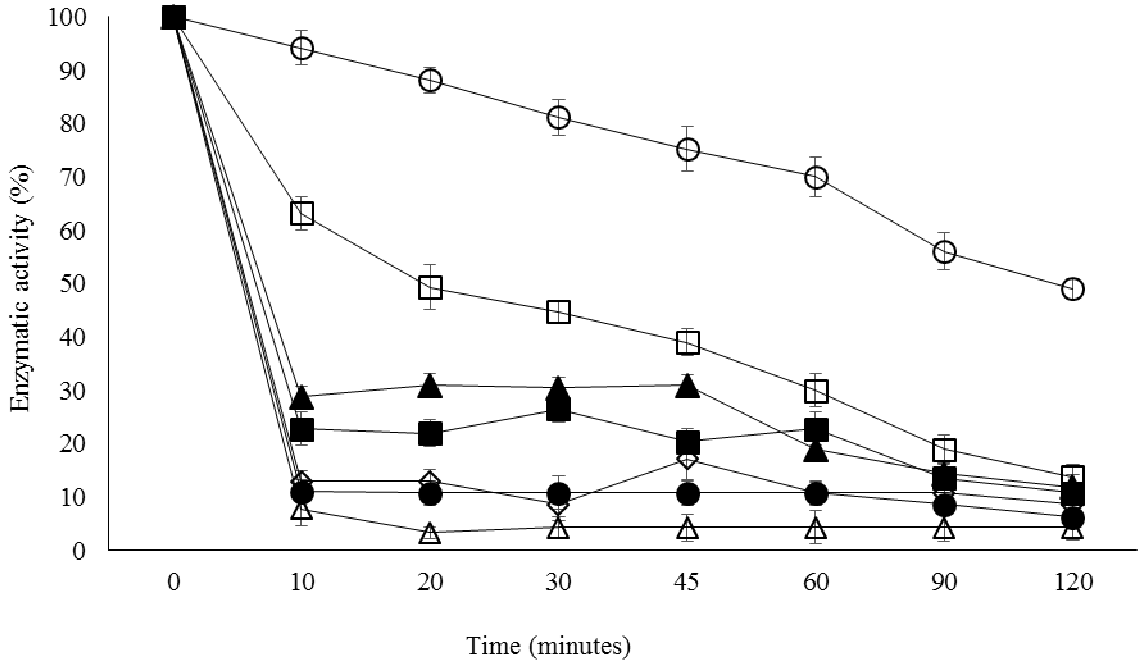

Fig 4. Thermal stability of cell-free supernatant produced by *P. fluorescens* PL5.4 at 40 °C (○), 50 °C (□), 60°C (▲), 70 °C (■), 80 °C (◊); 90 °C (●), 100 °C (Δ), in pre-incubation time of 10-120 minutes, with phosphate sodium buffer 0.2 M pH 7 using substrate azocasein. Results are expressed as % of activity enzymatic. Dates are means of triplicates.

4.3 Artigo 3: Evaluation of the adhesion potential by psychrotrophic bacteria isolated from refrigerated raw buffalo milk: simulating storage conditions

Evaluation of the adhesion potential by psychrotrophic bacteria isolated from refrigerated raw buffalo milk: simulating storage conditions

Marciéle Bogo[a], Géssica Aracéli Costa[a], Ana Paula Guedes Frazzon[a], Amanda de Souza da Motta[a]

[a] Departamento de Microbiologia, Imunologia e Parasitologia, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil.

Corresponding author: Amanda Motta. Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite, 500, sala 216. 90050-170. Porto Alegre, RS, Brazil. +5551 3308.4111. E-mail: amanda.motta@ufrgs.br

Abstract

Psychrotrophic bacteria in milk are of great concern to the dairy industry, because it can produce biofilm on a variety of surfaces. The aim of this study was to evaluate the in vitro adhesion surfaces by two psychrotrophic bacteria isolated from refrigerated raw buffalo milk. Initially, the strains were identified by molecular biology. After it was evaluated for biofilm formation on the stainless steel surface and polypropylene at temperatures of 7, 23 and 30 °C for 24, 48 and 72 hours of incubation. They were also tested for adhesion in stainless steel to 7 °C sterilized hole milk buffalo. Other tests was conducted with the strains adhesion in stainless steel in co-cultivation with strain *Staphylococcus* coagulase positive $A710^{-2}$, pre known as biofilm forming and isolated from buffalo milk. The co-cultive were tested in sterilized hole milk buffalo with stell surface at 7 °C. Isolates were identified by 16S rRNA gene sequencing considered *Pseudomonas fluorescens* PL5.4 e *Pseudomonas fluorescens* PL7.1. These psychrotrophic bacteria isolated from refrigerated raw buffalo milk were able adhere under the conditions tested, with counts of cells in biofilm exceeding 5,65 and 6,16 log CFU/cm^2 for stainless steel and polypropylene 6.3 and 6.37 log CFU/cm^2 respectively for *P. fluorescens* PL5.4 and *P. fluorescens* PL7.1. Both strains showed higher adhesion in polypropylene surface. The test stainless steel adhesion, to 7 °C sterilized buffalo milk, the tested strains showed higher counts to 4.85 and 5.09 log CFU/cm^2 for *P. fluorescens* PL5.4 and *P. fluorescens* PL7.1. Both psychrotrophic strains were able adhere in co-culture with the strain *Staphylococcus* coagulase positive $A710^{-2}$. This study shows that psychrotrophic bacteria originally isolated from refrigerated raw buffalo milk can adhere on surface stainless steel and polypropylene.

Keywords: Bacteria, adhesion, biofilm, contamination.

1. Introduction

Biofilms represent a negative impact on all of the dairy industry, as they may be present in all development stages of dairy products (Tan et al. 2014). Biofilms are defined as microbial cell aggregates embedded in a polymeric matrix composed of exopolysaccharides (EPS) connected to a biotic or abiotic surface. This bacterial aggregation is a phenomenon in which microorganisms interact with each other, may form a multi-species group (Abee et al. 2011).

Thus, the microorganisms can create a microenvironment, which enhances their survival in the most different surfaces (Teh et al. 2012). This type of organization has the advantage of promoting a high concentration of nutrients, ease of genetic exchange and greater ability to resist the lack of nutrients, pH and temperature changes, resisting further to cleaning procedures and treatments with antibiotics due to the protective effect of EPS (Nguyen and Yuk, 2013).

The biofilm is initiated by the presence of bacterial cells more organic and inorganic molecules deposited on the surface (Bridier et al. 2015). According to the study by Teh et al. (2012), the number of bacteria normally found in biofilms varies 10^4-10^8 CFU / cm^2. Then occurs the adhesion of microorganisms on the surface, can occur involving accessory structures of bacteria, such as fimbriae, forming a bridge between the bacterial cells and available substrate, promoting a consistent association (Kumar & Anand, 1998; Van Houdt & Michiels, 2010). After the initial adhesion, with microcolonies formed, develops into a mature biofilm that is associated with the production of exopolysaccharides by bacteria. The consolidation of biofilm architecture is believed to be related signaling mechanisms among bacteria, called quorum-sensing, as described in the literature (Blackledge et al. 2013; Soheili et al. 2015).

In the dairy industry there is the problem of the presence of milk residues on surfaces that can supply nutrients to the bacteria which are able to survive and proliferate (Teh et al. 2011). Microbial adhesion to surfaces of materials and equipment and subsequent biofilm formation is a major issue in the industry, since it goes to be a potential source of contamination. The spread of bacterial cells within the production environment, threatening the safety and quality of dairy products, resulting in diseases transmitted by food and economic losses (Carpentier and Cerf, 2011).

The presence of biofilms in the dairy industry is a risk to consumer health and damage to the dairy company because of the likelihood of spreading of pathogenic bacteria and spoilage and its by-products (toxins, proteases, lipases), leading to financial losses and standards of identity and quality of food (Burgess et al, 2010; Carpentier and Cerf, 2011).

An important group of forming biofilm microorganisms in the dairy industry, are psychrotrophic bacteria because they have the ability to grow at refrigerator temperature, between 4 °C and 10 °C, a condition in which the products are subjected (Pothakos et al. 2012; Neubeck et al. 2015). Although psychrotrophic bacteria account for less than 10% of the total flora of fresh milk in satisfactory sanitary conditions, this percentage can reach 75% in inadequate conditions for obtaining and processing (Nielsen, 2002). Besides its importance in the formation of biofilm, they have the potential for production of extracellular enzymes, both inside and outside of the biofilm, so that this structure becomes a proteases reservoir, for example (Marchand et al. 2012). Whereas the production of proteolytic enzymes there is a risk of the finished product, causing coagulation, gelation and deterioration of the yours organoleptic characteristics (Teh et al. 2011).

The objective of this study was to evaluate the potential of adhesion in stainless steel and polypropylene surfaces by psychrotrophic bacteria isolated from refrigerated raw buffalo milk, simulating storage conditions.

2. Materials and Methods

2.1 Bacterial cultures and cultivation conditions

This study investigates two cultures of proteolytic psychrotrophic bacteria, previously coded as PL5.4 and PL7.1. Both bacteria were previously isolated from samples of refrigerated raw buffalo milk. The cultures were initially kept frozen at -20 ° C in Tryptone Soy Broth (TSB Himedia, India) with 20% glycerol. For the reactivation of the isolates, it was used TSB with incubating the isolated at 30 °C for 48 hours and after it was made inoculation in culture Petry plate (Tryptone Soy Agar, TSA - Himedia, India) under the same conditions, to observe the purity of the isolates.

2.2 Molecular identification of psychrotrophic bacteria isolated from refrigerated raw buffalo milk

Total bacterial DNA was extracted as described by Donato (2007) with modifications. Cells were collected by centrifugation at 14.000 rpm for 4-5 minutes and resuspended in 40 µl of lysis solution containing 1 M NaOH, sodium dodecyl sulfate (SDS) at 10% and TE 1X buffer (Tris-HCl acid ethylenediaminetetraacetic (EDTA), pH 8.0), and this hurts prepared boiled at 100 °C for 15 minutes. After this, sample was diluted with 460 µl of TE 1X buffer homogenized and centrifuged at 14.000 rpm for 4-5 minutes, followed by removal of supernatant.

The 16S rRNA gene was amplified from genomic DNA by Polymerase Chain Reaction (PCR) using primers FC27 (5'-AGAGTTTGATCCTGGCTCAG-3') and R530 (5'-CCGCGGCTGCTGGCACGTA-3') (Gontang et al. 2007). The PCR reaction system consisted of 2 µl of DNA, 2.5 µl 1X buffer, 2.0 mM $MgCl_2$, 300 µM deoxynucleotide

triphosphates (dNTPs), 0.1 pM each primer, 1 U Taq DNA polymerase (Promega, Madison, WI, USA) and sterile distilled water to a final volume of 25 µl.

PCR amplification was performed using a Techne TC-5000 PCR Thermal Cycler under the following conditions: initial phase of denaturation at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 1 minutes, annealing temperature 58 °C for 1 minutes, 72 °C primer extension for 1 minute and finally a primer extension step at 72 °C for 5 minutes.

Aliquots of 30-60 ng of a PCR product of 503 bp and 4.5 pmol of each primer were subjected to automated sequencing (ABI PRISM 3500 Genetic Analyser-) by ACTGene Molecular Analysis Laboratory (Porto Alegre, Brazil). The sequences were identified using BLAST sequence nucleotides standard, using the 16S rRNA (bacteria and archaea) database optimized for highly similar sequences (Megablast).

2.3 Adherence of psychrotrophic bacteria isolated from refrigerated raw buffalo milk in stainless steel AISI 304 and polypropylene coupons

The ability of adherence of two isolates to stainless steel and polypropylene was investigated according to the methodology described by Nörnberg et al. (2011) and Bayoumi et al. (2012) with some modifications.

For the experiment, the colonies of bacteria at 24 hours growth were transferred to TSB culture medium and incubated at 30 °C for 24 hours. An aliquot of 1 ml was transferred to 9 ml of TSB and incubated at 30 °C for 24 hours with subsequent adjustment of the optical density (OD) using a spectrophotometer wavelength of 600 nm to 1,5 x 10^8 CFU / ml.

Stainless steel (AISI 304) coupons of 1 cm^2 were prepared by soaking in acetone for 30 minutes to remove any fat or fingerprints with subsequent washing with sterile distilled water and subjected to autoclave sterilization. Polypropylene coupons were cleaned and

sterilized by immersion in 70% ethanol then rinsed twice in sterile distilled water. For each isolate already prepared at a concentration of 1,5 x 10^8 CFU / ml, three coupons of each material were immersed in separate tubes inoculated with TSB medium. The tubes were then incubated without agitation at 7 °C, 23 °C and 30 °C for 24, 48, and 72 hours. Every 24 hours one coupon of each tube was aseptically removed using sterile tweezers, rinsed three times in sterile distilled water to remove non adherent cells. The coupons containing in adherent cells were re-immersed in 10 ml of 0.85% saline bath and subjected to ultrasonic machine (ultrasonic, USC 700, inique) with a frequency of 40 kHz, and treated for two periods 10 minutes order to release in adherent cells. Later dilutions were made of treatments which were subjected to plating on TSA and incubating at 30 °C for 24 hours to determine the number of in adherent cells, expressed as log CFU / cm^2.

Also, it determined the concentration of planktonic cells during the 72 hours incubation. Each 24 hours, an aliquot was withdrawn and decimal dilutions were made and plated in the same conditions. All counts were made in duplicate and each experiment was repeated twice.

2.4 Adherence of psychrotrophic bacteria isolated from refrigerated raw buffalo milk in stainless steel AISI 304 in sterile hole buffalo milk

For this experiment, we considered the procedures of the previous section 2.3. However, in order to simulate a real situation buffalo in stainless steel milk refrigerated tank storage, cultures of strains (PL5.4 and PL7.1) were incubated in the presence of the raw material milk.

New colonies of isolates were maintained on TSA transferred to the TSB broth culture medium and incubated at 30 °C for 24 hours. Then, an aliquot of 1 ml was transferred into 9

ml sterilized hole buffalo milk (121 °C for 10 minutes) and incubated at 30 °C for 24 hours, taking up an inoculum of approximately 1,5 x 10^8 CFU / ml.

The stainless steel coupons (AISI 304) 1 cm² were prepared as previously described in section 2.3. Three stainless steel AISI 304 coupons were immersed in tubes with buffalo milk, previously inoculated with the isolated separately. The tubes were incubated without agitation at a temperature of 7 °C for 72 hours. Every 24 hours a coupon of each tube was aseptically removed using sterile forceps and were washed and treated as described in item 2.3.

Counts were made on each of the coupons removed evaluating the amount of sessile and planktonic cells. All counts were performed in duplicate and each experiment was repeated twice.

2.5 Adherence of psychrotrophic bacteria in co-culture with *Staphylococcus* coagulase positive A710^{-2} isolated from refrigerated raw buffalo milk in stainless steel AISI 304 in sterile hole buffalo milk

In this test, we evaluated the ability of cultures strains (PL5.4 and PL7.1) to adhere on coupons stainless steel with another microorganism. Initially strains cultures had their standardized inoculum of 1,5 x 10^8 CFU / ml in sterilized buffalo milk, separately. Then, these were prepared adding the culture of *Staphylococcus* coagulase positive A710^{-2} (strain known as biofilm-forming) (1,5 x 10^8 CFU / ml) and mixed sterile hole buffalo milk. Three coupons stainless steel AISI (1 cm^2) were immersed in separate tubes inoculated with sterile hole buffalo milk and the cultures. The tubes were incubated at 7 °C and aliquots were collected for counting at 24, 48 and 72 hours. As a control, cultures were evaluated separately. The cell counts on the coupon and planktonic cells were performed according to the method described above (section 2.3) using the differential selective culture medium Mannitol Salt Agar (Himedia, India) for *Staphylococcus* coagulase positive A710^{-2} count and MacConkey

Agar (Himedia, India) for counting the *Pseudomonas* isolates. In addition, counts were made of recovered cells and planktonic cells. All counts were performed in duplicate and each experiment was repeated twice.

2.6 Statistical analysis

Three replicates were performed for each experiment, on two separate occasions. Statistic 12.5 Software was used for analysis of variance using ANOVA test with a critical probability of $P \leq 0,05$ followed by Tukey's test.

3. Results

3.1 Molecular identification of psychrotrophic bacteria isolated from refrigerated raw buffalo milk

Isolates were identified by 16S rRNA gene. The isolated previously encoded as PL5.4 and PL 7.1, were identified as *Pseudomonas fluorescens* PL5.4 with the code gb/KM579624.1 and *Pseudomonas fluorescens* PL7.1 with the code gb/JF327445.1 in Standard Nucleotide BLAST (available from http://www.ncbi.nlm.nih.gov). Both isolates had identity of at least 98% with a known database sequence.

3.2 Adherence of psychrotrophic bacteria isolated from refrigerated raw buffalo milk in stainless steel AISI 304 and polypropylene coupons

Cultures of psychrotrophic bacteria, previously identified as *P. fluorescens* PL5.4 and *P. fluorescens* PL7.1 were evaluated for *in vitro* adherence capacity at the temperatures of 7 °C, 23 °C and 30 °C for 24, 48 and 72 hours incubation.

A count of the number of planktonic cells from *P. fluorescens* PL5.4 at temperatures of 7 °C, 23 °C and 30 °C ranged from 8.12 to 8.39 log CFU / ml, 8.18 to 8.58 log CFU / ml

and 7.86- 8.64 log CFU / ml, respectively. For *P. fluorescens* PL7.1, the counts of planktonic cells were from 7.98 to 8.19 log CFU / ml, 7.74 to 8.48 log CFU / ml and 8.01 to 8.55 log CFU / ml for the temperatures of 7 °C, 23 °C and 30 °C respectively.

In the evaluation of sessile cells, it was observed that both cultures were able to adhere on both types of surface (stainless steel AISI 304 and polypropylene). The count of bacterial cells adherent on the surface of polypropylene were higher than stainless steel AISI 304 for both psychrotrophic isolates.

The number of adherent cells of *P. fluorescens* PL5.4 ranged from 5.31 to 6.37 log CFU / cm^2 on the surface of stainless steel AISI 304 and 6.3 to 7.44 log CFU / cm^2 for the polypropylene surface. In the stainless steel AISI 304 surface, it was not observed any significant difference between the times and the temperatures tested. However, the polypropylene surface showed differences depending on the treatment. The highest score was found with temperature of 30 °C with 72 hours incubation (Fig. 1-A).

The bacterial cell count of *P. fluorescens* PL7.1 adhered to the surface stainless steel AISI 304 ranged from 6.16 to 6.97 log CFU / cm^2, since these counts showed significant differences in relation to different times and temperatures tested. The bacterial cell count adhered at polypropylene surface ranged from 6,16 a 7,52 log CFU / cm^2 (Fig. 1-B).

3.3 Adherence of psychrotrophic strains in stainless steel AISI 304 coupons in sterile hole buffalo milk

This test aimed at assessing the ability of cells to adhere on stainless steel AISI 304 surfaces with sterilized buffalo milk presence at the temperature of 7 °C. The results of this test are shown in Fig. 2.

The count of bacterial cells adhered of *P. fluorescens* PL5.4 was 4.85 log CFU / cm^2 in 24 hours, 5.07 log CFU / cm^2 at 48 hours and 5.68 log CFU / cm^2 in 72 hours . For *P.*

fluorescens PL7.1 bacterial cell counts were 5.09 log CFU / cm^2 in 24 hours, 5.41 log CFU / cm^2 at 48 hours and 5.36 log CFU / cm^2 at 72 hours.

The higher count cell adhered on the surface of stainless steel AISI 304 was found in *P. fluorescens* PL5.4 in 72 hours of incubation, showing a significant difference from other counts. For the other two cultures tested, the count was greater at 72 hours of incubation.

The count of planktonic cells from strains of *P. fluorescens* PL5.4 and *P. fluorescens* PL7.1 ranged from 8.87 to 9.2 log CFU / ml and 8.87 to 9.54 log CFU / ml, respectively..

3.4 Adherence of psychrotrophic bacteria in co-culture with *Staphylococcus* coagulase positive $A710^{-2}$ in coupons stainless steel AISI 304 in sterile buffalo milk

In this study it was observed that both psychrotrophic bacteria were able to adhere on co-cultivation with *Staphylococcus* coagulase positive $A710^{-2}$ culture the stainless steel AISI 304 surface with sterile buffalo milk at a temperature of 7 °C.

The number of recovered bacterial cells of *P. fluorescens* PL5.4 after to cell adhered was 4.68 log CFU / cm^2 in 24 hours, 5.12 log CFU / cm^2 at 48 hours and 5.32 log CFU / cm^2 in 72 hours (Fig. 3.A). For *S. aureus* $A710^{-2}$ bacterial cell counts were 5.22 log CFU / cm^2 in 24 hours, of 5.26 log CFU / cm^2 at 48 hours and 5.48 CFU / cm^2 log at 72 hours (Fig. 4). The number of bacterial cells recovered from *P. fluorescens* PL5.4 was lower than in single culture, it was observed in *Staphylococcus* coagulase positive $A710^{-2}$. However, *Staphylococcus* coagulase positive A710-2 showed higher counts of in adhered cells bacterial on the surface stainless steel AISI 304 the than *P. fluorescens* PL5.4. Both showed no significant with 72 hours of incubation (Fig. 3.B).

Moreover, the reduction of the bacterial cell count of *S. aureus* $A710^{-2}$ and *P. fluorescens* PL5.4 in co-culture was also observed in planktonic cell count. The values range

from 6.63 to 7.54 log CFU / ml for *P. fluorescens* PL5.4 and from 6.16 to 7.31 log CFU / ml for *Staphylococcus* coagulase positive A710^{-2}.

The recovered bacterial cell counts of the stainless steel AISI 304 surface of *P. fluorescens* PL7.1 was 5.87 log CFU / cm^2 in 24 hours of 6.03 log CFU / cm^2 at 48 hours and 5.76 log CFU / cm^2 at 72 hours of culture (Fig. 5). In assessing the co-cultivation of *P. fluorescens* PL7.1 and *Staphylococcus* coagulase positive A710^{-2} lower in adhered cells than when grown in isolated form were observed. Since the culture of *Staphylococcus* coagulase positive A710^{-2} showed high counts of bacterial cells adhered to the stainless steel AISI 304 surface with respect to their individual cultivation. For *Staphylococcus* coagulase positive A710^{-2} the number of recovered cells was 6.19 log CFU / cm^2 in 24 hours, 6.41 log CFU / cm^2 at 48 hours and 6.36 log CFU / cm^2 at 72 hours of culture (Fig. 5). However, the association between cultures the *P. fluorescens* PL7.1 and *Staphylococcus* coagulase positive A710^{-2} showed a reduction in the number of bacterial cells adhered at 72 hours for evaluation over other times (Fig. 5).

It was also observed that co-culture *P. fluorescens* PL7.1 and *Staphylococcus* coagulase positive A710^{-2} stimulated the adhesion with increasing numbers of cell adhered of *Staphylococcus* coagulase positive A710^{-2}, while co-cultivation of *P. fluorescens* PL5.4 and *Staphylococcus* coagulase positive A710^{-2} showed a lower in adhered cells of *Staphylococcus* coagulase positive A710^{-2}. The count of planktonic cells in co-culture of *Staphylococcus* coagulase positive A710^{-2} and *P. fluorescens* PL7.1 also showed decline when compared with the simple culture of each isolate. Their scores ranged from 7.09 to 7.51 log CFU / ml for *P. fluorescens* PL7.1 and from 7.32 to 8.17 log CFU / ml for *Staphylococcus* coagulase positive A710^{-2} .

4. Discussion

The microbiota of raw milk is diverse and complex, due to its high nutritional content (Quigley et al. 2011). The species of the genus *Pseudomonas* are described as the main bacteria in refrigerated raw milk associated with biofilm formation (Abdallah et al. 2015) and the deterioration of milk and milk derivatives (De Jonghe et al. 2011; Cleto et al. 2012; Decimo et al. 2014; Scatamburlo et al. 2015; Pinto et al. 2015). The microorganism adhesion and biofilm formation is of great importance and can occur at any milk processing site from the milking even in the processing environment and the production of dairy products (Vlková et al. 2008; Hamadi et al. 2014). With the isolation of psychrotrophic bacteria from samples of refrigerated raw buffalo milk, this study can evaluate, identify and characterize these bacteria, and ability of *Pseudomonas* species to adhesion in different surfaces, temperatures (7, 23 and 30 °C) and incubation times (24, 48 and 72 hours). The studied sought to simulate the milk storage for a long time and with higher temperatures also exploring two contact surfaces: stainless steel AISI 304 and polypropylene. According to the results above, the strains of *P. fluorescens* PL5.4 and *P. fluorescens* PL7.1 can adhere on stainless steel AISI 304 surfaces and polypropylene, and a larger number of adhered cells could be observed on polypropylene surface to both isolates.

The fact that more cells are adhered on polypropylene surface can be due to the greater roughness characteristic by this surface. According to Dias et al. (2010) the macroscopic characteristics, and particularly the microscopic characteristics of surfaces are key to greater or lesser microbial adhesion, as the microtopography of the surface can make it more difficult for the cleaning procedures. Likewise, these micro-grooves or other imperfections create conditions for planktonic cells to adhere and form called biofilm.

Di Ciccio et al. (2015) also showed increased adhesion on polypropylene surface of the stainless steel surface. In addition, several authors have reported the ability of bacteria to form biofilms in many different materials usually employed in food processing environments,

such as in stainless steel, glass, rubber, polycarbonate, polyurethane, polystyrene, polypropylene, aluminum, titanium, and ceramics (Simões et al. 2010; Vázquez-Sánchez et al. 2013; Hamadi et al. 2014).

It was also observed that the two bacteria tested, *P. fluorescens* PL5.4 and *P. fluorescens* PL7.1, have the ability to adhere at different temperatures, indicating that once present in milk, may become a source of recontamination.

In the adhesion assay in stainless steel AISI 304, using sterilized buffalo milk at 7 °C, *P. fluorescens* PL5.4 and *P. fluorescens* PL7.1 showed adherence, with increasing number of cells which constituted this structure throughout the experimental time of 72 hours. These results show that these bacteria are an indication that a high amount of psychrotrophic bacteria in milk, may pose a problem for the dairy industry, since the biofilm made has a protective character to those present cells, creating one recontamination point, since the cleaning and disinfection process may become less effective.

The evaluation of the multi-species adherence in surface was also studied because this is considered the situation most commonly found in processing environments. To this, it was simulated growth and adhesion by co-cultivation of bacteria on stainless steel AISI 304 surfaces, with milk storage at 7 °C. All cultures employed demonstrated growth and biofilm formation under the conditions tested.

The co-culture *P. fluorescens* PL7.1 with *Staphylococcus* coagulase positive $A710^{-2}$ favored the accession of Gram-positive bacteria tested. According to Lindsay et al. (2002), Teh et al. (2012) exopolysaccharides production by Gram-negative bacteria favoring the setting of Gram-positive bacteria on stainless steel surfaces. Thus cells from several different species may coexist in a pre-formed biofilm. An example is a culture of *Listeria monocytogenes* and *Pseudomonas* biofilm in one (Marchand et al., 2012).

Since psychrotrophic bacteria can contaminate the milk from the milking and throughout the milk production chain, it is essential to adopt good practices of obtaining and manufacturing, making the cleaning process has become less chance of permanence of in biofilm cells and the formation of microbial biofilms (Huck et al. 2008; Latorre et al. 2010).

In conclusion, these microorganisms originally obtained from refrigerated buffalo milk have the potential to adhere on stainless steel AISI 304 surfaces with greater adhesion to surfaces of polypropylene. In addition, these psychrotrophic bacteria can grow and adhere in conditions simulating the refrigerated raw milk storage and in co-culture with other bacteria. These results reinforce the need for adequate milk storage temperature and time and care required to obtain good quality milk from its origin.

REFERENCE

Abdallah, M., Khelissa, O., Ibrahim, A., Benoliel, C., Heliot, L., Dhulster, P., Chihib, N. E., 2015. Impact of growth temperature and surface type on the resistance of *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilms to disinfectants. International Journal of Food Microbiology 214, 38-47.

Abee, T., Kovacs, A.T., Kuipers, O. P., Van Der Veen, S., 2011. Biofilm formationand dispersal in Gram-positive bacteria. Current Opinion in Biotechnology 22, 172-179.

Bayoumi, M.A., Kamal, R.M., Aal, S.F.A.E.A., Awad, E.I., 2012. Assessment of a regulatory sanitization process in Egyptian dairy plants in regard to the adherence of some food-borne prthogens and theis biofilms. International Journal of Food Microbiology 158, 225-231.

Blackledge, M.S., Worthington, R.J., Melander C., 2013. Biologically inspired strategies for combating bacterial biofilms. Current Opinion in Pharmacology 13: 699-706.

Bridier, A., Sanchez-Vizuete, P., Guilbaud, M., Piard, J.C., Naïtali, M., Briandet, R., 2015. Biofilm-associated persistence of food-borne pathogens. Food Microbiology 45, 167-178.

Burgess, S.A., Lindsay, D., Flint, S.H., 2010. Thermophilic bacilli and their importance in dairy processing. International Journal of Food Microbiology 144, 215–225.

Carpentier, B., Cerf, O., 2011. Review - Persistence of *Listeria monocytogenes* in food industry equipment and premises. International Journal of Food Microbiology 145, 1-8.

Cleto, S., Matos, S., Kluskens, L., Vieira, M.J., 2012. Characterization of contaminants from a sanitized milk processing plant. PLoS ONE 7, e40189.

Decimo, M., Morandi, S., Silvetti, T., Brasca, M., 2014. Characterization of Gram-negative psychrotrophic bacteria isolated from Italian bulk tank milk. Journal of Food Science 79, M2081-M2090.

De Jonghe, V., Coorevits, A., Van Hoorde, K., Messens, W., Landschoot, A. V., De Vos, P., Heyndrickx, M., 2011. Influence of storage conditions on the growth of *Pseudomonas* species in refrigerated raw milk. Applied and Environmental Microbiology 77, 460- 470.

Dias, N.A.A., Santos Junior, A.C., Caixeta, D., Domingues, A.S., Piccoli, R.H., Alves, E. 2010. Eletromicrografias de cupons de aço inoxidável, polietileno e vidro aderidas com bactérias *Listeria monocytogenes*. XIX Congresso de Pós-Graduação da UFLA.

Di Ciccio, P., Vergara, A., Festino, A.R., Paludi, D., Zanardi, E., Ghidini, S., Ianieri A. 2015. Biofilm formation by Staphylococcus aureus on food contact surfaces: Relationship with temperature and cell surface hydrophobicity. Food Control 50: 930-936.

Donato, S.T., Sidrim, J.J.C. 2007 Comparação de métodos convencionais e semi-automatizados para identificação de Enterococcus spp. frente a Biologia Molecular em identificações discrepantes. Dissertação (Mestrado). Universidade Federal do Ceará, Faculdade de Medicina, Fortaleza, Brasil.

Gontang, E.A., Fenical, W., Jesen, P.R., 2007. Phylogenetic diversity of Gram-positive bacteria cultured from merine sediments. Applied and Environmental Microbiology 73, 3272-3282.

Hamadi, F., Asserne, F., Elabed, S., Bensouda, S., Mabrouki, M., Latrache, H., 2014. Adhesion of *Staphylococcus aureus* on stainless steel treated with three types of milk. Food Control 38, 104-108.

Huck, J. R., Sonnen, M., & Boor, K. J., 2008. Tracking heat-resistant, cold-thriving fluid milk spoilage bacteria from farm to packaged product. Journal of Dairy Science 91, 1218-1228.

Kumar, C.G., Anand, S.K., 1998. Significance of microbial biofilms in food industry: a review. International Journal of Food Microbiology 42, 9-27.

Lindsay, D., Brözel, V. S., Mostert, J. F., Von Holy, A., 2002. Differential efficacy of a chlorine dioxide-containing sanitizer against single species and binary biofilms of dairy-associated *Bacillus cereus* and a *Pseudomonas fluorescens* isolate. Journal of Applied Microbiology 92, 352–361.

Latorre, A. A., Van Kessel, J. S., Karns, J. S., Zurakowski, M. J., Pradhan, A. K., Boor, K. J., Jayarao, B. M., Houser, B. A., Daugherty, C. S., Schukken, Y. H., 2010. Biofilm in milking equipment on a dairy farm as a potential source of bulk tank milk contamination with Listeria monocytogenes. Journal of Dairy Science 93, 2792-2802.

Marchand, S., De Block, J., De Jonghe, V., Coorevits, A., Heyndrickx, M., Herman, L., 2012. Biofilm formation in milk production and processing environments; influence on milk quality and safety. Comprehensive Reviews in Food Science and Food Safety 11, 133-147.

Neubeck, M.V., Baur, C., Krewinkel, M., Stoeckel, M., Kranz, B., Stressler, T., Fischer, L., Hinrichs, J., Scherer, S., Wenning, M., 2015. Biodiversity of refrigerated raw milk microbiota and their enzymatic spoilage potential. International Journal of Food Microbiology 211, 57–65.

Nguyen, H.D.N., Yuk, H.G., 2013. Changes in resistance of *Salmonella* Typhimurium biofilms formed under various conditions to industrial sanitizers. Food Control 29: 236-240.

Nielsen, S.S., 2002. Plasmin System and Microbial Proteases in Milk: Characteristics, Roles, and Relationship. Journal Agricultural and Food Chemistry 50, 6628-6634.

Nornberg, M.F.B.L., Mentges, M.L., Silveira, S.T., Tondo, E.C, BrandelIi, A.A., 2011. Psychrotrophic *Burkholderia cepacia* strain isolated from refrigerated raw milk showing proteolytic activity and adhesion to stainless steel. Journal of Dairy Research 78, 257-262.

Pinto, C. L. O., Machado, S. G., Martins, M. L., Vanetti, M. C. D., 2015. Identifcation of proteolytic psychrotrophic bacteria isolated from refrigerated raw milk and characterization of its spoilage potential. Revista do Instituto de Laticínios Cândido Tostes 70, 105-116.

Pothakos, V., Samapundo, S., Devlieghere, F. 2012. Total mesophilic counts underestimate in many cases the contamination levels of psychrotrophic lactic acid bacteria (LAB) in chilled-stored food products at the end of their shelf-life. Food Microbiology 32: 437-443.

Quigley, L., O'Sullivan O., Ross, R. P., Fitzgerald, G. F., Cotter, P. D., 2011. Molecular approaches to analysing the microbial composition of raw milk and raw milk cheese. International Journal of Food Microbiology 150, 81-94.

Scatamburlo, T. M., Yamazi, A. K., Cavicchioli, V. Q., Pieri, F. A., Nero, L. A., 2015. Spoilage potential of *Pseudomonas* species isolated from goat milk. Journal of Dairy Science 98, 759-764

Simões, M., Simões, L. C., & Vieira, M. J., 2010. A review of current and emergent biofilm control strategies. LWT-Food Science Technology 43,573-583.

Soheili, V., Bazzaz, B.S.F., Abdollahpour, N., Hadizadeh, F., 2015. Investigation of Pseudomonas aeruginosa quorum-sensing signaling system for identifying multiple inhibitors using molecular docking and structural analysis methodology. Microbial Pathogenesis 89: 73-78.

Tan, S.Y., Chew, S.C., Tan, S.Y.; Givskov, M.; Yang, Y., 2014. Emerging frontiers in detection and control of bacterial biofilms. Current Opinion in Biotechnology 26, 1-6.

Teh, K.H., Flint, S., Palmer, J., Lindsay, S., Andrewes, P., Bremer, P., 2011. Thermo-resistant enzyme-producing bacteria isolated from the internal surfaces of raw milk tankers. International Dairy Journal 21, 742-747.

The, K.H., Flint, S., Palmer, J, Andrewes, P., Bremer, P., Lindsay, D., 2012. Proteolysis produced within biofilms of bacterial isolates from raw milk tankers. International Journal of Food Microbiology 157, 28-34.

Van Houdt, R., Michiels, C.W. 2010. Biofilm formation and the food industry, a focus on the bacterial outer surface. Journal of Applied Microbiology, 109, 1117-1131.

Vázquez-Sánchez, D., Habimana, O., Holck, A., 2013. Impact of food-related environmental factors on the adherence and biofilm formation of natural *Staphylococcus aureus* isolates. Current Microbiology 66,110-121.

Vlková, H., Babák, V., Seydlová, R., Pavlík, I., Schlegelová, J., 2008. Biofilms and hygiene on dairy farms and in the dairy industry: sanitation chemical products and their effectiveness on biofilms - a review. Czech Journal of Food Sciences 26, 309-323.

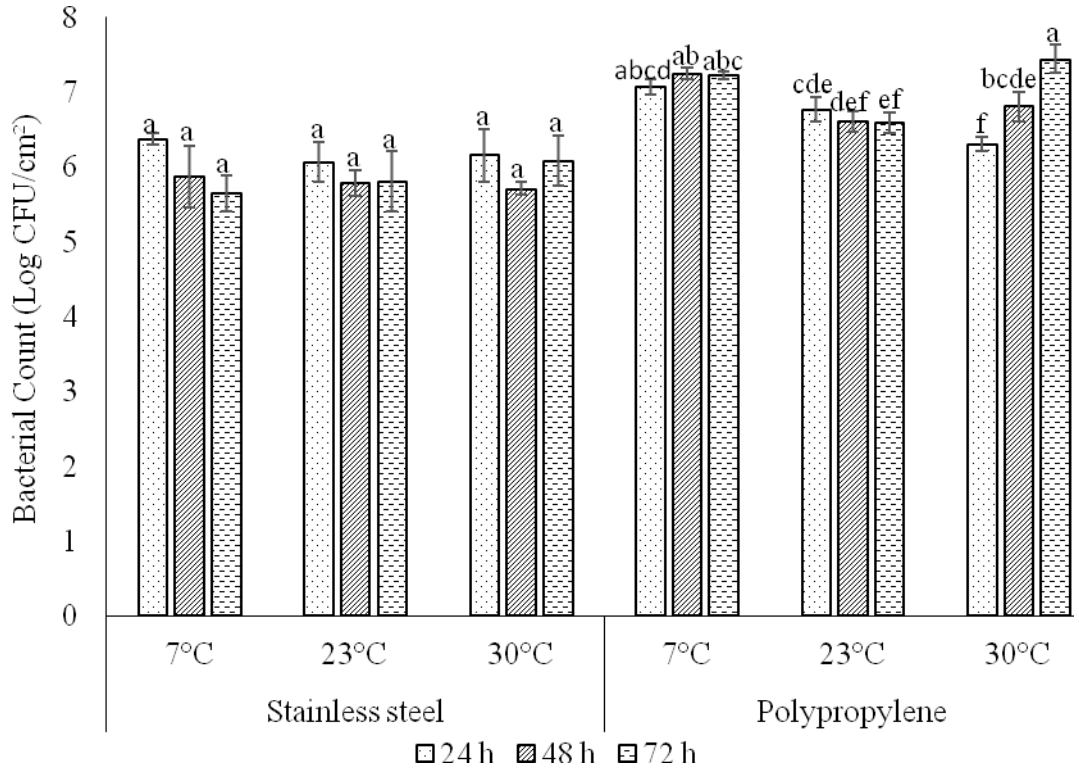

Fig. 1.B. Biofilm formation on stainless steel AISI 304 and polypropylene by strain *P. fluorescens* PL5.4 in temperature of the 7°, 23° and 30 °C for 24, 48 and 72 hours.

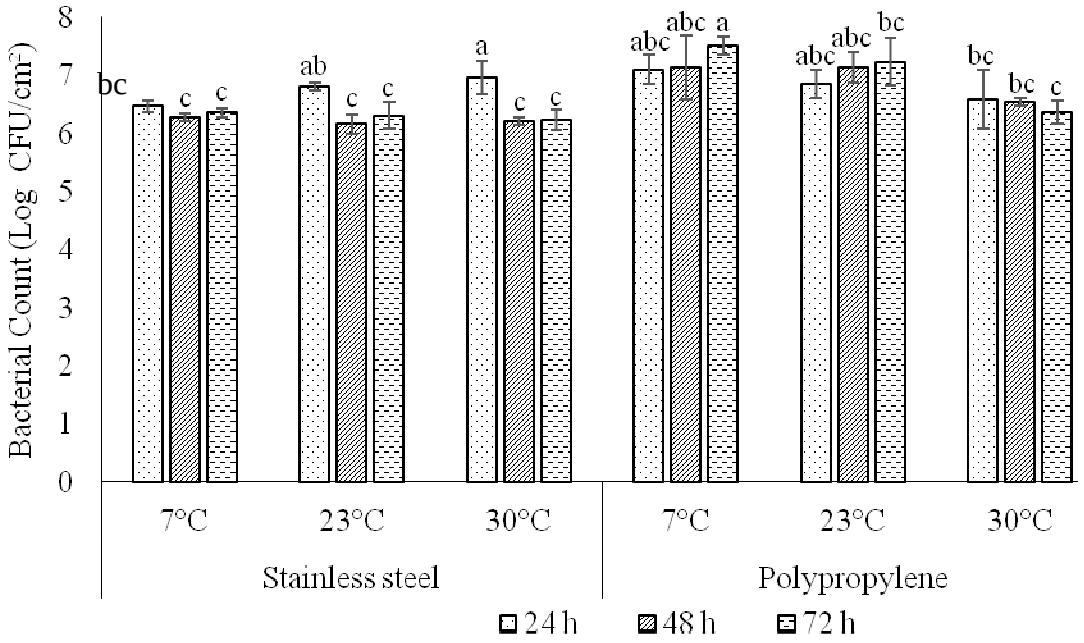

Fig. 1.B. Biofilm formation on stainless steel AISI 304 and polypropylene by strain *P. fluorescens* PL7.1 in temperature of the 7°, 23° and 30 °C for 24, 48 and 72 hours.

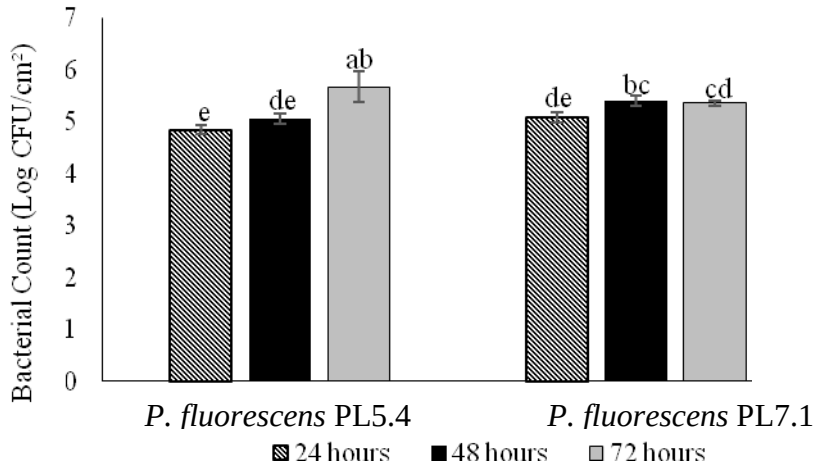

Fig. 2.A. Biofilm formation of the strains *P. fluorescens* PL5.4 and *P. fluorescens* PL7.1 in stainless steel AISI 304, with milk culture medium at a temperature of 7 °C for 24, 48 and 72 hours.

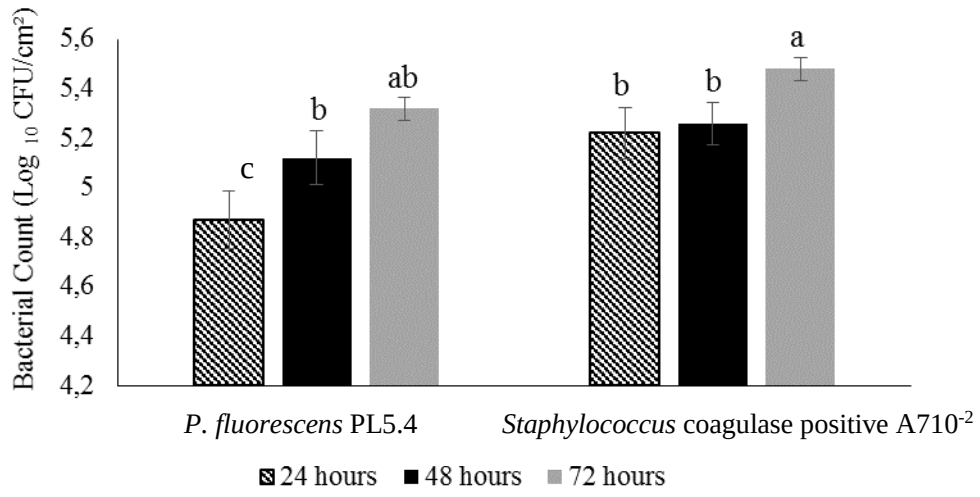

Fig. 3.A. Biofilm formation of *P. fluorescens* PL5.4 strain and co-cultivation with *Staphylococcus* coagulase positive A710^{-2} in stainless steel AISI 304 in sterile buffalo milk medium at a temperature of 7 °C for 24, 48 and 72 hours.

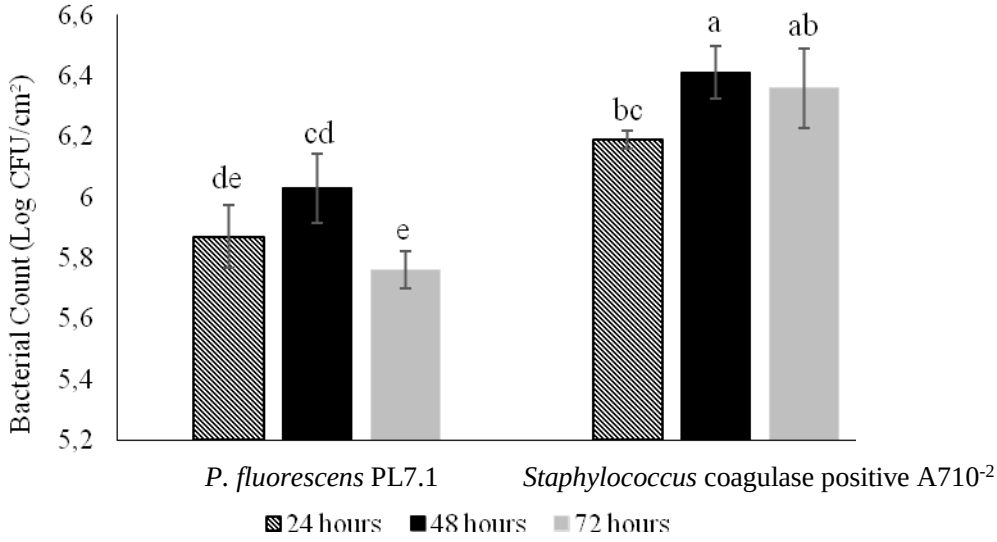

Fig. 3.B. Biofilm formation of *P. fluorescens* PL7.1 strain and co-cultivation with *S Staphylococcus* coagulase positive A710^{-2} in stainless steel AISI 304 in sterile buffalo milk medium at a temperature of 7 °C for 24, 48 and 72 hours.

5. RESULTADOS E DISCUSSÃO GERAL

O aumento da produção do leite de búfala no Brasil, remete a uma preocupação com a qualidade microbiológica dos produtos elaborados a base dessa matéria-prima (CUNHA NETO et al., 2005; RICCI & DOMINGUES, 2012).

No Brasil não há legislação que defina os padrões de identidade de qualidade do leite bubalino. Dessa forma, o beneficiamento do leite cru de búfala segue a legislação para leite bovino (BRASIL, 2011).

A IN 62 – MAPA indica o tempo de armazenamento de no máximo 48 horas e a uma temperatura máxima de conservação do leite na propriedade rural de 7 ℃ e de no máximo 10 ℃ no estabelecimento proces sador. A falta de cuidados com as condições de higiene pode favorecer o crescimento de microrganismos psicrotróficos, que mesmo em temperaturas baixas podem continuar multiplicando-se (NIELSEN, 2002; PINTO et al., 2006; POTHAKOS et al., 2012; VITHANAGE et al., 2014).

O leite cru, devido ao seu alto teor nutricional, é um meio de cultivo que pode conter uma população microbiana diversa e complexa (AMARAL et al., 2005; QUIGLEY et al., 2011; VACHEYROU et al., 2011). Com essas características, o leite pode ser facilmente deteriorado, representando uma grande preocupação para a indústria de lácteos, que se preocupa com a garantia da qualidade microbiológica e físico-química de seus produtos (GUERREIRO et al., 2005).

Poucos estudos da microbiota psicrotrófica de leite cru de búfala tem sido realizado quando comparado com o leite bovino. Grande parte das investigações realizadas sobre a microbiota psicrotrófica são do leite cru bovino, e estas incluem principalmente espécies Gram-negativas (MARTINS et al., 2006; DUFOUR et al., 2008; DECIMO et al., 2014; MACHADO et al., 2015). No presente estudo realizado foi observado, também, um predomínio de bactérias Gram-negativas nas amostras de leite cru de búfala refrigerado, principalmente o gênero *Pseudomonas*.

O estudo de Silva et al. (2011) evidenciou a presença desse gênero. Os autores verificaram um elevado nível de bactérias do gênero *Pseudomonas* no leite cru bovino recém-ordenhado (5,70 log UFC / ml), e uma diferença significativa após 48 horas de armazenamento a 7 ℃ (6,14 log UFC / ml). Assim, como o estudo de

Castro (2012), onde 48,75% das cepas isoladas de leite cru refrigerado encontradas, foram identificadas como pertencentes ao gênero *Pseudomonas*.

Quanto ao perfil de resistência dos isolados psicrotróficos, em três ou mais classe de antibióticos, foi observada em 12 (57,14%) dos isolados analisados, demostrando perfil de resistência a múltiplas drogas. Cinco desses isolados com múltipla resistência são do gênero *Pseudomonas*.

As bactérias psicrotróficas, além de crescerem em baixas temperaturas (4-10 ℃), possuem a capacidade de produzir enzimas extracelulares como proteases, lipases e lecitinases (NÖRBERG et al., 2010; MARQUES et al., 2012; VITHANAGE et al., 2014). Os resultados encontrados no presente estudo, avaliando as propriedades enzimáticas dos 21 isolados de bactérias psicrotróficas, demonstraram essas as culturas produziram proteólise em ágar leite bovino e bubalino. Além disso, a atividade lipolítica e a produção de lecitinase foi evidenciada por nove e sete bactérias psicrotróficas, respectivamente. E ainda, verificou-se que quatro das 21 bactérias psicrotróficas apresentaram combinação de atividades proteolítica, lipolítica e lecitinase, podendo comprometer ainda mais a qualidade do produto final.

Essas enzimas bacterianas podem ter efeito prejudicial nos produtos lácteos durante o armazenamento prolongado, podendo reduzir ou alterar as propriedades físico-químicas do produto, causando assim defeitos na funcionalidade e propriedades sensoriais (CHEN et al., 2003; MARTINS 2006; NÖRBERG et al., 2011).

Muitas dessas enzimas bacterianas são termorresistentes, permanecendo ativas sobre uma ampla faixa de temperaturas mesmo após tratamento térmico. Processos como a pasteurização e Ultra-High Temperature (UHT) não são suficientes para inativar as enzimas bacterianas (MARCHAND et al., 2009; NÖRBERG et al., 2010; TEH et al., 2011; BAGLINIÈRE et al., 2013). Por exemplo, a protease termoresistente produzida por espécies de *Pseudomonas* e *Bacillus* podem permanecer ativas depois do processamento de pasteurização lenta (63.5 ℃ durante 30 minutos) (NABRDALIK et al., 2010; TEH et al., 2011).

Proteases termoresistentes produzidas por bactérias psicrotróficas têm sido relatadas por promoverem a coagulação e a gelificação das proteínas do leite. O estudo de Vidal-Martins et al. (2005) demonstra a ação da enzima protease em leite UHT, onde foi acompanhada a vida de prateleira verificando possíveis

alterações de viscosidade. Os autores constataram que houve aumento da proteólise no decorrer do armazenamento e aumento da viscosidade aparente após 60 dias de estocagem, provavelmente relacionados à presença de proteases de bactérias psicrotróficas do leite cru.

A termorresistência também foi evidenciada em nosso estudo, onde cinco cepas psicrotróficas apresentaram atividade proteolítica termorresistente a temperatura de pasteurização lenta (63.5 ℃ por 30 minutos) aplicada na indústria para elaboração de queijos mozzarella de leite de búfala. Dessas cepas bacterianas, quatro pertencem ao gênero *Pseudomonas*. Esses resultados corroboram com outros estudos da literatura onde a maior parte das investigações realizadas sobre produção de enzimas proteolíticas relatam o gênero *Pseudomonas* (BUTTON et al., 2011; BAGLINIÈRE et al., 2013; DECIMO et al., 2014; VITHANAGE et al., 2014; MACHADO et al., 2015). A coagulação promovida pela proteólise termorresistênte produzida pelas cinco cepas foi evidenciada no leite bovino e bubalino. Ao final de cindo dias de observação, verificou-se que a enzima proteolítica das cinco bactérias psicrotróficas coagularam totalmente ambos os leites testado. Dessa forma as bactérias psicrotróficas podem, efetivamente, causar degradação de produtos lácteos.

O sobrenadante livre de células da cepa *Pseudomonas fluorescens* PL5.4 apresentou altos valores na atividade enzimática e resistência térmica nos testes. A partir desses resultados, o sobrenadante livre de células deste isolado foi escolhido para melhor entendimento do perfil enzimático. O resultado dessa caracterização parcial mostrou um sobrenadante livre de células com maior atividade enzimática em pH 7, temperatura ótima de 40 ℃, mostrou-se resistentes aos detergentes e solventes testados e apresentou-se sensível aos sais: cloreto de cobre ($CuCl_2$), cloreto de cobalto ($CoCl_2$) e sulfato de zinco ($ZnSO_4$). Contudo, com os inibidores de proteases usados, não foi possível identificar o tipo de protease produzida por este isolado. Porém, proteases do gênero Pseudomonas tem sido identificada como metaloproteases (Woods et al. 2001; RAJ et al. 2012). Mais estudos devem ser realizados para a purificação e caracterização desta enzima. Importante destacar que o perfil enzimático dessas bactérias implica na deterioração de produtos lácteos, dando um sabor amargo e gelificação durante seu armazenamento.

Assim como as enzimas hidrolíticas, também encontramos o biofilme microbiano como um dos problemas mais significativos para o setor leiteiro, quanto à

segurança microbiológica e a qualidade físico-química desta matéria-prima. A presença de biofilmes em superfícies de equipamentos parece ser um dos principais focos de contaminação de produtos lácteos por bactérias patogênicas e deteriorantes (OLIVEIRA et al., 2010).

As bactérias psicrotróficas avaliadas no presente estudo, demostram que fatores como temperatura e presença de nutrientes em superfícies implicam diretamente sobre a formação de biofilmes microbianos. Observou-se que as cepas psicrotróficas em determinadas condições de temperaturas e na presença de resíduos de nutriente (resíduos de leite bovino e bubalino) formaram biofilme com determinada intensidade, e quando alteradas essas condições não formaram biofilme ou formaram em menor intensidade. Esses resultados corroboram com o estudo de Martin (2015), onde as condições de tempo e temperatura foram determinantes para a formação de biofilme, assim como para a ação de sanitizantes.

Nesta avaliação de formação de biofilme, as bactérias psicrotróficas foram classificadas como forte, moderadas, fracas ou não formadoras de biofilme. Não houve entre as cepas, uma forte formadora de biofilme, no entanto, as cepas *Pseudomonas fluorescens* PL5.4 e *P. fluorescens* PL7.1 foram as que se destacaram quanto a capacidade de formação de biofilme, aparecendo em mais tratamentos como moderadas formadoras destas estruturas.

Dessa forma, essas duas cepas foram identificadas por métodos moleculares sendo então denominadas *P. fluorescens* PL5.4 e *P. fluorescens* PL7.1. Ainda, essas cepas foram escolhidas para serem testadas para a formação de biofilme em superfícies de aço inoxidável e polipropileno, em diferentes tempos e temperaturas de incubação. Os resultados dessa avaliação demostraram que para ambas as bactérias psicrotróficas houve maior formação de biofilme na superfície de polipropileno.

Apesar da superfície de aço inoxidável ser uma superfície de fácil adesão pelas bactérias em função da hidrofobicidade (BOARI, 2008), a superfície de polipropileno teve a maior quantidade de células bacterianas formadoras de biofilme, o que pode ser explicado pela rugosidade apresentada por essa superfície. Dias et al. (2010), afirmam que as características macroscópicas e microscópicas da superfície determinam maior ou menor formação de biofilme, pois os micro-sulcos e outras imperfeições podem criar condições para as células planctônicas formarem biofilme.

Contudo, a superfície de polipropileno é pouco utilizada na indústria de lácteos, onde a maioria dos equipamentos são compostos por aço inoxidável. Assim, as superfícies de tanques de resfriamento, tubulações, válvulas e tanques de transporte são frequentemente apontadas como fontes de contaminação devido à formação de biofilme (SHI & ZHU, 2009).

Os problemas causados por biofilmes em superfícies de aço inoxidável se agravam quando se trata de superfícies de troca de calor, pois a taxa de transferência de calor em um processo de controle biológico pode ser reduzida, como a pasteurização, promovendo ineficácia (SANTOS et al., 2011a; SANTOS et al., 2011b). Ou ainda, os biofilmes podem comprometer as condições dos equipamentos aumentando a taxa de corrosão de suas superfícies (MANSFELD, 2007; MIGUEL et al., 2014).

Referente ao binômio tempo / temperatura analisado no estudo, foi possível observar para a cultura *P. fluorescens* PL5.4, em aço inoxidável e nas temperaturas de 7, 23 e 30 ℃, que as contagens de células bacterianas em biofilme após 24 horas não apresentaram diferença significativa. Isto demostra que, mesmo em temperaturas baixas, a formação de biofilme não foi afetada e que o armazenamento prolongado pode manter a formação de biofilme.

Quanto ao isolado *P. fluorescens* PL7.1, as contagens das células bacterianas em biofilme na superfície de aço inoxidável, a 7, 23 e 30℃, foram significativas e com as maiores contagens de células em 24 horas de incubação. Não houve diferença significativa entre 48 e 72 horas de incubação, demostrando a manutenção do biofilme. Já em polipropileno, as temperaturas de 7 ℃ e 23 ℃ apresentaram interferência positiva sobre a formação de biofilme, pois o isolado *P. fluorescens* PL7.1 apresentou maiores contagens de células bacterianas em biofilme nessas condições. Demostrando preferência por temperaturas mais baixas. Ainda, foi possível verificar na superfície de polipropileno, em 7 ℃ com 72 horas de incubação a maior contagem de células em biofilme.

Levando em consideração que as bactérias psicrotróficas do estudo foram isoladas de leite cru de búfala refrigerado de tanque de armazenado de aço inoxidável, se essas bactérias estivessem em alta quantidade, podem formar biofilme nesse equipamento nas temperaturas de 7, 23 e 30 ℃ já com 24 horas de armazenamento, e se manteriam por 48 e 72 horas de armazenamento.

A capacidade apresentada por esses isolados psicrotróficos de formar biofilme nessas condições pode comprometer a qualidade e a segurança do produto final, visto que o biofilme apresenta um carácter de proteção para as células presentes, tornando o processo de limpeza e desinfecção ineficaz, criando um ponto de recontaminação.

Outro aspecto que precisa ser considerado no ambiente de obtenção e processamento de leite, é o comum desenvolvimento de biofilmes polimicrobianos e o favorecimento das interações sinérgicas da coexistência microbiana em biofilme que impactam a distribuição bacteriana (SIMÕES et al., 2010; SREY et al., 2013).

Considerando esse fato, o presente trabalho também contemplou a formação de biofilme por mais de uma espécie, verificando a interação na formação de biofilme das bactérias psicrotróficas (*P. fluorescens* PL5.4 e *P. fluorescens* PL7.1) com uma cultura de *Staphylococcus aureus* A710^{-2}, também isolada de leite cru de búfala refrigerado e previamente selecionada como formadora de biofilme. As bactérias psicrotróficas foram capazes de formar biofilme em conjunto com o *S. aureus* A710^{-2}. O co-cultivo de *P. fluorescens* PL7.1 e *S. aureus* A710^{-2} simulando as condições de armazenamento do leite de búfala a 7 °C, favoreceu a formação de biofilme da bactéria Gram-positiva (*S. aureus* A710^{-2}). Assim, diferentes espécies bacterianas podem coexistir formando biofilme, como por exemplo a cultura de *Listeria monocytogenes* e *Pseudomonas* em um biofilme (MARCHAND et al., 2012).

Como relatado nos resultados, os isolados psicrotróficos de leite cru de búfala refrigerado possuem a capacidade de produzir enzimas proteolíticas e de formarem biofilme. De acordo com Teh et al. (2014), o potencial de produção de enzimas proteolíticas dentro do biofilme é pouco correlacionado no setor de lácteos como uma fonte de contaminação de produtos lácteos.

Nosso estudo encontrou a produção de enzimas proteolíticas dentro do biofilme dos isolados de *P. fluorescens* PL5.4 e *P. fluorescens* PL7.1. Contudo, foi encontrada uma maior atividade proteolítica nas mesmas células na forma planctônica. Nossos achados não estão de acordo com o estudo de Teh et al. (2012), onde a atividade enzimática proteolítica foi maior dentro do biofilme.

Nesse contexto, a produção de enzimas hidrolíticas extracelulares, principalmente proteolíticas e lipolíticas, a formação de biofilmes bacterianos e, ainda a produção de enzimas proteolíticas dentro do biofilme na propriedade rural e

na indústria representam uma fonte de contaminação e deterioração tanto da matéria-prima quanto para produto acabado.

A ocorrência de uma alta quantidade de bactérias psicrotróficas está diretamente relacionada com a qualidade da matéria-prima e com as condições higiênicas da ordenha. Dessa forma, práticas mais eficientes para impedir e limitar o desenvolvimento de bactérias psicrotróficas devem ser adotadas, visando à redução de perdas decorrentes da deterioração e à diminuição dos riscos à saúde dos consumidores.

6. CONCLUSÕES

As bactérias psicrotróficas isoladas de leite cru refrigerado de búfala apresentaram atividade proteolítica, nove produziram atividade lipolítica e sete foram produtoras de lecitinase. A combinação das três atividades enzimáticas foi demostrada por quatro cepas.

O perfil de resistência dos isolados psicrotróficos, em mais de três classe de antibióticos, foi observada em 57,14% dos isolados analisados.

A termorresistência das enzimas proteolíticas foi evidenciada por cinco bactérias psicrotróficas. As enzimas foram capazes de promover a coagulação total do leite de bubalino e bovino após cinco dias de observação.

O sobrenadante livre de células obtido a partir do cultivo de *P. fluorescens* PL5.4 mostrou-se com maior atividade enzimática em pH 7 e uma temperatura ótima de 40 ℃.

A formação de biofilme pelas culturas psicrotróficas foi evidenciada por todas as cepas em pelo menos uma das condições em que foram testadas. As cepas *P. fluorescens* PL5.4 e *P. fluorescens* PL7.1 se destacaram entre as demais, pois foram moderadas formadoras de biofilme em mais tratamentos em que foram testadas.

A avaliação da formação de biofilme em corpos de prova, mostrou maior adesão pelas cepas *P. fluorescens* PL5.4 e *P. fluorescens* PL7.1 em superfície de polipropileno. Ainda, ambas as bactérias psicrotróficas foram capazes de crescer e formar biofilme em co-cultivo com a cultura de *S. aureus* $A710^{-2}$, dessa forma, podendo coexistirem em biofilmes.

Foi possível detectar a atividade proteolítica dentro do biofilme produzida pelas cepas *P. fluorescens* PL5.4 e *P. fluorescens* PL7.1. Contudo, a atividade proteolítica foi maior quando as células estavam em condições planctônicas.

Dessa forma, o potencial enzimático e de formação de biofilme apresentados pelas bactérias psicrotróficas representam uma fonte de contaminação e deterioração do leite e derivados, causando problemas para a economia do setor lácteo e a saúde pública.

REFERÊNCIAS BIBLIOGRAFICAS

Abdallah M, Khelissa O, Ibrahim A, Benoliel C, Heliot L, Dhulster P, Chihib N. 2015. Impact of growth temperature and surface type on the resistance of Pseudomonas aeruginosa and Staphylococcus aureus biofilms to disinfectants. International Journal of Food Microbiology. 214: 38-47.

Abee T, Kovacs AT, Kuipers OP, Van Der Veen S. 2011. Biofilm formationand dispersal in Gram-positive bacteria. Current Opinion in Biotechnology. 22: 172-179.

Ahmad S, Anjum FM, Huma N, Sameen A, Zahoor T. 2013. Composition and physico-chemical characteristics of buffalo milk with particular emphasis on lipids, proteins, minerals, enzymes and vitamins. The Journal of Animal and Plant Sciences. 23: 62-74.

Alves EC, Dahmer AM, Borges AF. 2014. Total bacterial count and somatic cell count in refrigerated raw milk stored in communal tanks. Brazilian Journal of Food Technology. 17: 221-225.

Amaral FR; De Carvalho LB, Da Silva N, Brito JRF. 2005. Qualidade do leite de búfalas: composição. Revista Brasileira de Reprodução Animal, Belo Horizonte. 29: 106-110.

Andrighetto C, Jorge AM, Gomes MIFV, Hoch A, Piccinin A. 2005. Efeito da Monensina Sódica sobre a Produção e Composição do Leite, a Produção de Mussarela e o Escore de Condição Corporal de Búfalas Murrah. Revista Brasileira Zootecnia. 34, 641-649.

Araújo KBS, Rangel AHN, Fonseca FCE, Aguiar EM, Simplício AA, Novaes LP, Lima Júnior, DM. 2012. Influence of the year and calving season on production, composition and mozzarella cheese yield of water buffalo in the State of Rio Grande do Norte, Brazil. Italia Journal of Animal Science. 11: 86-91.

Arcuri EF, Silva PDL, Brito MAVP, Brito JRF, Lange CC, Magalhães MMA. 2008. Contagem, isolamento e caracterização de bactérias psicrotróficas contaminantes de leite cru refrigerado. Ciência Rural. 38, 2250-2255.

Baglinière F, Matéos A, Tanguy G, Jardin J, Briard-Bion V, Rousseau F, Robert B, Beaucher E, Gaillard JL, Amiel C, Humbert G, Dary A, Gaucheron F. 2013. Proteolysis of ultra high temperature-treated casein micelles by AprX enzyme from *Pseudomonas fluorescens* F induces their destabilization. International Dairy Journal. 31: 55-61.

Barreto MLJ, Rangel AHN, Araújo VM, Bezerra KC, Medeiros HR, Oliveira JPF, Andarde KD. 2010. Análise de correlação entre a contagem de células somáticas (ccs), a produção, o teor de gordura, proteína e extrato seco total do leite bubalino. Agropecuária Científica no Semiárido. 6: 47-53.

Braun P, Sutherland JP. 2005. Predictive modeling of growth and measurement of enzymatic synthesis and activity by a cocktail of selected Enterobacteriaceae and *Aeromonas hydrophila*. International Journal of Food Microbiology. 105: 257-266.

Baur C, Krewinkel M, Kranz B, Neubeck MV, Wenning M, Scherer S, Stoeckel M, Hinrichs J, Stressler T, Fischer L. 2015. Quantification of the proteolytic and lipolytic activity of microorganisms isolated from raw milk. International Dairy Journal. 49: 23-29.

Behlau I, Gilmore MS. 2010. Microbial Biofilms in Ophtalmology and Infections Disease. Archives of Ophtalmology, 126 (11), 1572-1581.

Bekker A, Jooste P, Steyn L, Bothma C, Hugo A, Hugo C. 2016. Lipid breakdown and sensory analysis of milk inoculated with *Chryseobacterium joostei* or *Pseudomonas fluorescens*. International Dairy Journal. 52: 101-106.

Boari CA. **Formação de biofilme em aço inoxidável por *Aeromonas hydrophila* e *Staphylococcus aureus* sob diferentes condições de cultivo**. Tese (Ciência dos Alimentos) - Universidade Federal de Lavras, Lavras.

Boulangé-Petermann L, Jullien C, Dubois PE, Benezech T, Faille C. 2004. Influence of surface chemistry on the hygienic status of industrial stainless steel. Biofouling. 20: 25-33.

Brasil. Ministério da Agricultura, Pecuária e Abastecimento – MAPA. **Bovinos e Bubalinos**. 2010. Brasília, BRASIL-MAPA; 2010.
Disponivel em: <http://www.agricultura.gov.br/animal/especies/bovinos-e-bubalinos>. Acesso em 31/01/2015.

Brasil. Ministério da Agricultura, Pecuária e Abastecimento – MAPA, **Instrução Normativa Nº 62, de 29 De Dezembro de 2011, Regulamento Técnico de Produção, Identidade e Qualidade do Leite tipo A, o Regulamento Técnico de Identidade e Qualidade de Leite Cru Refrigerado, o Regulamento Técnico de Identidade e Qualidade de Leite Pasteurizado e o Regulamento Técnico da Coleta de Leite Cru Refrigerado e seu Transporte a Granel**. 2011. Brasília, BRASIL: MAPA: 2011

Bremer PJ, Fillery S, Mcquillan AJ. 2006. Laboratory scale clean-in-place (CIP) studies on the effectiveness of different caustic and acid wash steps on the removal of dairy biofilms. International Journal of Food Microbiology. 106: 254-262.

Bridier A, Sanchez-Vizuete P, Guilbaud M, Piard JC, Naïtali M, Briandet R. 2015. Biofilm-associated persistence of food-borne pathogens. Food Microbiology. 45: 167-178.

Buchon L, Laurent P, Gounot AM, Guespin-Michel JF. 2000. Temperature dependence of extracellular enzymes production by psychrotrophic and psychrophilic bacteria. Biotechnology Letters. 22: 1577-1581.

Burgess SA, Lindsay D, Flint SH. 2010. Thermophilic bacilli and their importance in dairy processing. International Journal of Food Microbiology. 144: 215–225.

Button PD, Roginski H, Deeth HC, Craven HM. 2011. Improved shelf life estimation of UHT milk by prediction of proteolysis. Journal of Food Quality. 34: 229–235

Carpentier B, Cerf O. 2011. Review - Persistence of *Listeria monocytogenes* in food industry equipment and premises. International Journal of Food Microbiology. 145: 1–8.

Castro MSR. 2012. ***Enterococcus* spp e *Pseudomonas* spp isolados de ambiente de processamento de produtos lácteos: identificação, formação de biofilmes multi-espécies e controle por agentes sanitizantes.** Tese (Doutorado em Tecnologia de Alimentos) - Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas, Campinas.

Chen L, Daniel RM, Coolbear T. 2003. Detection and impact of protease and lipase activities in Milk and milkpowders. International Dairy Journal. 13: 255-275.

Chen TR, Wei QK, Chen YJ. 2011. *Pseudomonas* spp. and *Hafnia alvei* growth in UHT milk at cold storage. Food Control. 22: 697-701.

Cheng C, Zhang Z, Chen S, Bryersand JD, Jiang S. 2007. Inhibition of bacterial adhesion and biofilm formation on zwitterionic surfaces. Biomaterials, Amsterdam. 28: 4192-4199.

Coelho KO, Machado PF, Coldebella A, Cassoli LD, Corassin CH. 2004. Determinação do perfil físico-químico de amostras de leite de búfalas, por meio de analisadores automatizados. Ciência Animal Brasileira. 5: 167-170.

Cunha Neto OC, Oliveira CAF, Hotta RM, Sobral PJA. 2005. Avaliação do iogurte natural produzido com leite de búfala contendo diferentes níveis de gordura. Ciência e Tecnologia de Alimentos, Campinas. 3: 448-453.

Damé MCf, De Lima CT, Marcondes CR, Ribeiro MER, Garner ADV, Santos CS. 2010. Production and quality of buffalo milk in Rio Grande do Sul: Preliminary data. Embrapa Clima Temperado. 1-46.

Davey ME, O'Toole GA. 2000. Microbial Biofilms: from ecology to molecular genetics. Microbiology and Molecular Biology Reviews. 64: 847-867.

Decimo M, Morandi S, Silvetti T, Brasca M. (2014). Characterization of Gram-negative psychrotrophic bacteria isolated from Italian bulk tank milk. Journal of Food Science. 79: M2081-M2090.

Deeth, HC. 2006. Lipoprotein lipase and lipolysis in Milk. International Dairy Journal. 16: 555-562.

De Jonghe V, Coorevits A, Van Hoorde K, Messens, W, Landschoot AV, De Vos P, Heyndrickx M. 2011. Influence of storage conditions on the growth of Pseudomonas species in refrigerated raw milk. Applied and Environmental Microbiology 77: 460- 470.

De Lima TCC, Rangel AHN, Macêdo CS, De Araújo TPM, De Araújo VM, De Lima Júnior DM; Murmann L, Novaes LP. 2014. Composição e qualidade do leite e do soro do leite de búfalas no estado do rio grande do norte. Acta Veterinaria Brasilica. 8: 25-30.

Dias NAA., Santos Junior AC, Caixeta D, Domingues AS, Piccoli RH, Alves E. 2010. Eletromicrografias de cupons de aço inoxidável, polietileno e vidro aderidas com bactérias *Listeria monocytogenes*. XIX Congresso de Pós-Graduação da UFLA.

Di Ciccio P, Vergara A, Festino A.R., Paludi D., Zanardi E., Ghidini S., Ianieri A. 2015. Biofilm formation by Staphylococcus aureus on food contact surfaces: Relationship with temperature and cell surface hydrophobicity. Food Control 50: 930-936.

Dufour D, Nicod` eme M, Perrin C, Driou A, Brusseaux E, Humbert G, Gaillard JL, Dary A. 2008. Molecular typing of industrial strains of Pseudomonas spp. isolated from milk and genetical and biochemical characterization of an extracellular protease produced by one of them. International Journal of Food Microbiology. 125: 188-96.

Eneroth A, Ahrné S, Molin G. 2000. Contamination routes of Gram-negative spoilage bacteria in the production of pasteurized milk, evaluated by randomly amplified polymorphic DNA (RAPD). International Dairy Journal. 10: 325-331.

Ercolini D, Russo F, Ferrocino I, Villani F. 2009. Molecular identification of mesophilic and psychrotrophic bacteria from raw cow's milk. Food Microbiology. 26: 228–231.

Fairbairn DJ, Law BA. 1986. Proteinases of psychrotrophic bacteria: Their production, properties, effects and control. Journal of Dairy Research.53: 139-177.

FAO. FAOSTAT. Food and Agriculture Organization of the United Nations. Disponívelem:<http://faostat.fao.org/faostat/collections?Version=ext&hasbulk=0>. Acesso em: 31/01/2015.

Faria GHF, Vieira DAP, Machado SS. 2008. Comparação da composição do leite em diferentes espécies: uma revisão. I Simpósio Educação Tecnologia E Sociedade, CEFET-GO.104-108.

Flemming H, Wingender J. 2010. The biofilm matrix. Nature Reviews. 8: 623-633.

Frølund B, Griebe T, Nielsen PH. 1995. Enzymatic activity in the activatedsludge floc matrix. Applied Microbiology and Biotechnology. 43: 755-761.

Gamarra NN, Villena GK, Gutiérrez-Correa M. 2010. Cellulase production by *Aspergillus niger* in biofilm, solid-state, and submerged fermentations. Applied Microbiology and Biotechnology. 87: 545-551.

Gândara ALN, De Oliveira JS. 2000. Adesão de linhagem selvagem de *Streptococcus thermophilus* em superfície de aço inoxidável e efeitos da higienização na sua remoção. Ciências e Tecnologia de Alimententos. 20.

Giaouris E, Chorianopoulos N, Doulgeraki A, Nychas GJ. 2013. Co-Culture with Listeria monocytogenes within a dual-species biofilm community strongly increases resistance of *Pseudomonas putida* to benzalkonium chloride. PloS One. 8: e77276.

Guerreiro PK, Machado MRF, Braga GC, Gasparino E, Franzener ASM. 2005. Qualidade microbiológica de leite em função de técnicas profiláticas no manejo de produção. Ciência e Agrotecnologia. 29: 216-222.

Gürler Z, Kuyucuoğlu Y, Pamuk S. 2013. Chemical and microbiological quality of Anatolian Buffalo milk. African Journal of Microbiology Research. 7: 1512-1517.

Gutiérrez D, Delgado S, Vázquez-Sánchez D, Martínez B, Cabo ML, Rodríguez A, Herrera JJ, García P. 2012. Incidence of *Staphylococcus aureus* and analysis of associated bacterial communities on food Industry surfaces. Applied and Environmental Microbiology. 78: 8547-8554.

Habimana O, Møretrø T, Langsrud S, Vestby LK, Nesse LL, Heir E. 2010. Micro ecosystems from feed industry surfaces: A survival and biofilm study of S*almonella* versus host resident flora strains. BMC Veterinary Research. 6: 48-58,

Haddadi K, Moussaoui F, Hebia I, Laurent F, Le Roux Y. 2005. *E. coli* proteolytic activity in milk and casein breakdown. Reproduction Nutrition Development. 45: 485-496.

Han B, Meng Y, Li M, Yang Y, Ren F, Zeng Q, Nout RMJ. 2007. A survey on the microbiological and chemical composition of buffalo milk in China. Food Control. 18: 742-746.

Han X, Lee FL, Zhang L, Guo MR. 2012. Chemical composition of water buffalo milk and its low-fat symbiotic yogurt development. Funtional foods in Health and Disease. 4: 86-106.

Hashmi S, Saleem Q. 2015. An investigation on microbiological and chemical quality of buffalo milk supplies. International Journal of Current Microbiology and Applied Science. 4: 78-83.

Holm C, Jepsen L, Larsen M, Jespersen L. 2004. Predominant microflora of downgraded danish bulk tank milk. Journal of Dairy Science, Champaing. 87: 1151-1157.

Hussain I, Yan J, Grandison AS, Bell AE. 2012. Effects of gelation temperature on Mozzarella-type curd made from buffalo and cows' milk: 2. Curd yield, overall quality and casein fractions. Food Chemistry. 135: 1404-1410.

Jayamanne VS, Adams MR. 2004. Survival of probiotic bifidobacteria in buffalo curd and their effect on sensory properties. International Journal of Food Science and Technology. 39: 719-725.

Joseph B, Otta SK, Karunasagar I, Karunasagar I. 2001. Biofilm formation by *Salmonella* spp. on food contact surfaces and their sensitivity to sanitizers. International Journal of Food Microbiology. 64: 367-372.

Lamagna B, Pasolini MP, Nizza S, Mallardo K, Formicola M, Costagliola A, Fatone G, Fiorito F, Paciello O, Martino L. 2015. Conjunctival cytological examination, bacteriological culture, and antimicrobial resistance profiles of healthy Mediterranean buffaloes (*Bubalus bubalis*) from Southern Italy. Asian Pacific Journal of Tropical Biomedicine. 5: 889-895.

Lei C, Peng Z, Day T, Xinping Y, Bai X, Yuan C. 2011. Experimental observation of surface morphology effect on crystallization fouling in plate heat exchangers. International Commications in Heat and Mass Transfer. 38: 25-30.

Machado SG, Bazzolli DMS, Vanetti MCD. 2013. Development of a PCR method for detecting proteolytic psychrotrophic bacteria in raw Milk. International Dairy Journal. 29: 8-14.

Machado SG, Da Silva LF, Bazzolli DMS, Heyndrickx M, Costa PMA, Maria Cristina Vanetti MCD. 2015. *Pseudomonas* spp. and *Serratia liquefaciens* as predominant spoilers in cold raw milk. Journal of Food Science. 80: M1842-M1849.

Mansfeld F. 2007. The interaction of bacteria and metal surfaces. Electrochimica
Acta. 52(27): 7670-7680.

Marchand S, De Block J, De Jonghe V, Coorevits A, Heyndrickx M, Herman L. 2012. Biofilm formation in milk production and processing environments; Influence on milk quality and safety. Comprehensive Reviews in Food Science and Food Safety. 11: 133-147.

Marchand S, Vandriesche G, Coorevits A, Coudijzer K, De Jonghe V, Dewettinck E, Paul De Vos P, Devreese B, Heyndrickx M, Block J. 2009. Heterogeneity of heat-resistant proteases from milk Pseudomonas species. International Journal of FoodnMicrobiology. 133: 68-77.

Marino M, Frigo F, Bartolomeoli I, Maifreni M. 2011. Safety-related properties of staphylococci isolated from food and food environments. Journal of Applied Microbiology. 110: 550-561.

Marques SC, Evangelista SR, Piccoli RH. (2012). Diversity of psychrotrophic bacteria isolated from the milk cooling collective tanks and antibiotics resistance profile of the microorganisms isolates. Revista Instituto Adolfo Lutz. 71: 670-676.

Martin JGP. 2015. **Biofilmes de Staphylococcus aureus isolados de laticínios produtores de queijo Minas frescal**. Tese (Ciência e Tecnologia de Alimentos) Universidade de São Paulo Escola Superior de Agricultura “Luiz de Queiroz”, Piracicaba.

Martins ML, Pinto CLO, Rocha RB, De Araújo EF, Vanetti MCD. 2006. Genetic diversity of Gram-negative, proteolytic, psychrotrophic bacteria isolated from refrigerated raw milk. International Journal of Food Microbiology. 111: 144-148.

Mcinnis EA, Kalanetra KM, Mills DA, Maga EA. 2015. Analysis of raw goat milk microbiota: Impact of stage of lactation and lysozyme on microbial diversity. Food Microbiology. 46: 121-131.

Miguel EM, De Carvalho AF, De Andrade NJ, Pires ACS, De Paula JCJ, Costa RGB. 2014. Formação de biofilmes em trocadores de calor e seus efeitos em leite e derivados. Revista Instito Laticínios Cândido Tostes. 69: 53-63.

Millogo V, Svennersten KS, Ouédraogo GA, Agenäs S 2010. Raw milk hygiene at farms, processing units and local markets in Burkina Faso. Food Control. 21: 1070–1074.

Minard O, Ahmad S, Rousseau F, Briard-Bion V, Gaucheron F, Lopez C. 2010. Buffalo vs. cow milk fat globules: size distribution, zeta-potential, compositions in total fatty acids and in polar lipids from the milk fat globule membrane. Food Chemistry. 120: 544-551.

Morita Y, Komoda E, Ono K, Kumagai S. 2011. Survival of biofilm-forming Salmonella on stainless steel bolt threads under dry conditions. Shokuhin eiseigaku zasshi. Journal of the Food Hygienic Society of Japan. 52: 299-303.

Nabrdalik M, Grata K, Latala A. (2010). Proteolytic activity of Bacillus cereus streins. Proceedings of ECOpole. 4: 273-276.

Neubeck MV, Baur C, Krewinkel M, Stoeckel M, Kranz B, Stressler T, Fischer L, Hinrichs J, Scherer S, Wenning M. 2015. Biodiversity of refrigerated raw milk microbiota and their enzymatic spoilage potential. International Journal of Food Microbiology. 211: 57-65.

Nguyen HDN, Yuk HG. 2013. Changes in resistance of *Salmonella* Typhimurium biofilms formed under various conditions to industrial sanitizers. Food Control 29: 236-240.

Nicodème M, Grill JP, Humbert G, Gaillard JL. 2005. Extracellular protease activity of different *Pseudomonas* strains: dependence of proteolytic activity on culture conditions. Journal of Applied Microbiology. 99: 641-648.

Nielsen SS. 2002. Plasmin System and Microbial Proteases in Milk: haracteristics, Roles, and Relationship. Journal Agricultural and Food Chemistry. 50: 6628-6634.

Nörnberg MFBL, Tondo EC, Brandelli A. 2009. Bactérias psicrotróficas e atividade proteolítica no leite cru refrigerado. Acta Scientiae Veterinariae. 37: 157-163.

Nörnberg MBL, Friedrich RSC, Weiss RDN, Tondo EC, Brandelli A. 2010. Proteolytic activity among psychrotrophic bacteria isolated from refrigerated raw milk. International Journal of Dairy Technology. 63: 41-46.

Nörnberg MBL, Mentges ML, Silveira ST, Tondo EC, Brandelli A. 2011. A psychrotrophic *Burkholderia cepacia* strain isolated from refrigerated raw milk showing proteolytic activity and adhesion to stainless steel. Journal of Dairy Research. 78: 257-262.

Oliveira MMM, Brugnera DF, Piccoli RH. 2010. Biofilmes microbianos na indústria de alimentos: uma revisão. Revista do Instituto Adolfo Lutz. 69: 277-284.

Olivieri DA. **Avaliação da qualidade microbiológica de amostras de mercado de queijo mussarela, elaborado a partir de leite de búfala (Bubalus bubalis)**. (2004) Dissertação (Mestrado), Piracicaba. Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo. 61 f.

Oosthuizen MC, Steyn B, Lindsay D, Brözel VS, Von AH. 2001. Novel method for the proteomic investigation of a dairy-associated Bacillus cereus biofilm. FEMS Microbiology Letters. 194: 47-51.

Palmer J, Flint S, Brooks J. 2007. Bacterial cell attachment, the beginning of a biofilm. Journal of Industrial Microbiology and Biotechnology. 34: 577-588.

Parque SH, Cheon HL, Parque KH, Chung MS, Choi SH, Ryu S, Kang DH. 2012. Inactivation of biofilm cells of foodborne pathogen by aerosolized sanitizers. International Journal of Food Microbiology. 154: 130-134.

Perin LM, Moraes PM, Almeida MV, Nero LA. 2012.Intereference of storage temperatures in the development of mesophilic, psychrotrophic, lipolytic and proteolytic microbiota of raw Milk. Ciências Agrárias, Londrina. 33: 333-342.

Pinto CLO, Martins M L, Vanett MCD. 2006. Qualidade microbiológica de leite cru refrigerado e isolamento de bactérias psicotroficas proteolíticas. Ciência e Tecnologia de Alimentos. 26: 645-651.

Pothakos V, Samapundo S, Devlieghere F. 2012. Total mesophilic counts underestimate in many cases the contamination levels of psychrotrophic lactic acid bacteria (LAB) in chilled-stored food products at the end of their shelf-life. Food Microbiology. 32: 437-443.

Quigley L, O'Sullivan O, Beresford TP, Ross RP, Fitzgerald FG, Cotter PD. 2011. Molecular approaches to analyzing the microbial composition of raw milk and raw milk cheese. International Journal of Food Microbiology. 150:81–94.

Quigley L, O'sullivan O, Stanton C, Beresford TP, Ross RP, Fitzgerald GF, Cotter PD. 2013. The complex microbiota of raw milk. FEMS Microbiology Rev. 37: 664-698.

Raj A, Khess N, Pujari N, Bhattacharya S, Das A, Rajan SS. 2012. Enhancement of protease production by *Pseudomonas aeruginosa* isolated from dairy effluent sludge and determination of its fibrinolytic potential Asian Pacific Journal of Tropical Biomedicine. S1845-S1851.

Ricci GD, Domingues PF. 2012. O leite de búfala. Revista de Educação Continuada em Medicina Veterinária e Zootecnia do CRMV-SP / Journal of Continuing Education in Animal Science of CRMV-SP. 10: 14–19.

Samarzija D, Zamberlin S, Pogacic T. 2012. Psychrotrophic bacteria and their negative effects on milk and dairy products quality. Mljekarstvo. 62: 77-95.

São Paulo (Estado). Secretaria de Agricultura e Abastecimento. Resolução SAA nº 24 de 01 de agosto. 1994: **Dispõe sobre as normas técnicas de produção e classificação dos produtos de origem animal e as relativas às atividades de fiscalização e inspeção dos produtos de origem animal.** Cap.7, Artigo 134. [1994?] Disponível em: <http:/ cda.sp.gov.br / legislacao/>. Acesso em: 12 abr. 2015.

Santos AL, Pereira DBC, Saraiva CB. 2011a. A formação do Fouling e seus impactos no processamento e limpeza de indústrias de laticínios (Parte 1). Revista Leite e Derivados.125: 54-65.

Santos PA, Silva MAP, Moreira, GN, Barros JC, Oliveira AN, Nicolau ED. 2011b. Evolução da proteólise do leite inoculado in vitro com *Pseudomonas fluorescens*. Boletim CEPPA. 28(2): 313-320.

Schembri MA, Kjærgaard K, Klemm P. 2003. Global gene expression in *Escherichia coli* biofilms. Molecular Microbiology. 48: 253-267.

Schwering M, Song J, Louie M, Turner RJ, Ceri H. 2013. Multi-species biofilms defined from drinking water microorganisms provide increased protection against chlorine disinfection. Biofouling, v. 29, p. 917–928.

Sheehan JJ, Patel DA, Drake MA, Mcsweeney PLH. 2009. Effect of partial substitution of bovine for caprine milk on the compositional, volatile, non-volatile and sensory characteristics of sem-hard cheeses. International Dairy Journal. 19: 498-509.

Shi X, Zhu X. 2009. Biofilm formation and food safety in food industries. Trends Food Science & Technology. 20: 407-413.

Silva MR, Sacanavacca J, Gandra AKV, Seixas FA, Gandra EA. 2011. Avaliação higiênico-sanitária do leite produzido em Umuarama (Paraná). Boletim do CEPPA. 28 (2) 271-280.

Simões M, Simões LC, Vieira MJ. 2010. A review of current and emergent biofilm control strategies. LWT- Food Science and Techonology. 43: 573-583.

Soares AD, Rangel AHN, Novaes LP, De Lima Júnior DM, Bezerra KC. 2013. Composição do leite de búfala em diferentes ordens de parto. Agropecuária Científica no Semi-Árido. 9: 53-60.

Sofos JN, Geornaras I. 2010. Overview of current meat hygiene and safety risks and summary of recent studies on biofilms, and control of *Escherichia coli* O157:H7 in non intact, and *Listeria monocytogenes* in ready-to-eat,meat products.Meat Science. 86: 2-14.

Soheili V, Bazzaz BSF, Abdollahpour N, Hadizadeh F. 2015. Investigation of Pseudomonas aeruginosa quorum-sensing signaling system for identifying multiple inhibitors using molecular docking and structural analysis methodology. Microbial Pathogenesis. 89: 73-78.

Sorhaug T, Stephaniak L. 1997. Psychrotrophs and their enzymes in milk and dairy products: quality aspects. Trends in Food Science and Technology. 8: 35-41.

Souza EL, Meira QGS, Barbosa IM, Atheyde AJAA, Da Conceição ML, Sirqueira Junior JP. 2014. Biofilm formation by Staphylococcus aureus from food contact surfaces in a meat-based broth and sensitivity to sanitizers. Brazilian Journal of Microbiology. 45: 67-75.

Srey S, Jahid IK, Há S-D. 2013. Biofilm formation in food industries: A food safety concern. Food Control. 31: 572-585.

Stoodley P, Sauer K, Davies DG, Costerton JW. 2002. Biofilme as complex differentiated communities. Annun Rev Microbiology. 56: 187-209.

Swai ES, Schoonman L. 2011. Microbial quality and associated health risks of raw milk marketed in the Tanga region of Tanzania. Asian Pacific Journal of Tropical Biomedicine. 1: 217-222.

Takahashi H, Suda T, Tanaka Y, Kimura B. 2010. Cellular hydrophobicity of *Listeria monocytogenes* involves initial attachment and biofilm formation on the surface of polyvinyl chloride. Letters in Applied Microbiology. 50: 618-625.

Tang L, Pillai S, Revsbech NP, Schramm A, Bischoff C, Meyer RL. 2011. Biofilm retention on surfaces with variable roughness and hydrophobicity. Biofouling. 27: 111-121.

Teh KH, Flint S, Palmer J, Lindsay D, Andrewes P, Bremer P. 2011. Thermo-resistant enzyme-producing bacteria isolated from the internal surfaces of raw milk tankers. International Dairy Journal. 21: 742-747.

Teh KH, Flint S, Palmer J, Andrewes P, Bremer P, Lindsay D. 2012. Proteolysis produced within biofilms of bacterial isolates from raw milk tankers. International Journal of Food Microbiology. 157: 28-34.

Teh KH, Lindsay D, Palmer J, Andrewes P, Bremer P, Flint S. 2013. Lipolysis within single culture and co-culture biofilms of dairy origin. International Journal of Food Microbiology. 163: 129-135.

The KH, Flint S, Palmer J, Andrewes P, Bremer P, Lindsay D. 2014. Biofilm - An unrecognised source of spoilage enzymes in dairy products? International Dairy Journal, 34: 32-40.

Tondo EC, BARTZ S. 2012. **Microbiologia e Sistemas de Gestão da Segurança de Alimentos**. Editora Sulina. Porto Alegre.

Toyofuku M, Roschitzki B, Riedel K, Eberl L. 2012. Identification of proteins associated with the *Pseudomonas aeruginosa* Biofilme extracelular matrix. Journal Protoeme Research. 11: 4906-4915.

Vacheyrou M, Normand AC, Guyot P, Cassagne C, Piarroux R, Bouton Y. 2011. Cultivable microbial communities in raw cow milk and potential transfers from

stables of sixteen French farms. International Journal of Food Microbiology. 146:253–62.

Vidal-Martins AMC, Salotti BM, Rossi Junior OD, Penna ALB. 2005. Evolução do índice proteolítico e do comportamento reológico durante a vida de prateleira de leite UAT/UHT. Ciências Tecnologia de Alimentos, Campinas. 25: 698-704.

Vithanage NR, Yeager TR, Jadhav SR, Palombo EA, Datta N. 2014. Comparison of identification systems for psychrotrophic bacteria isolated from raw bovine milk. International Journal of Food Microbiology. 189: 26-38.

Verruma MR, Salgado JM. 1994. Análise química do leite de búfala em comparação ao leite de vaca. Scientia Agricola Piracicaba. 51: 131-137.

Van Houdt R, Michiels CW. 2010. Biofilmformation and the food industry, a focus on the bacterial outer surface. Journal of Applied Microbiology. 109: 1117-1131.

Woods RG, Burger M, Beven CA, Beacham IR. 2001. The aprX–lipA operon of *Pseudomonas fluorescens* B52: a molecular analysis of metalloprotease and lipase production. Microbiology. 147, 345–354.

Zhang S, Lv J (2014) Purification and properties of heat-stable extracelular protease from *Pseudomonads fluorescens* BJ-10. Food Sci Technol. 51: 1185–1190.