UFRRJ

INSTITUTO DE TECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA E TECNOLOGIA DE ALIMENTOS

TESE

Atividade Proteolítica de Bactérias Psicrotróficas Isoladas de Leite Cru e Implicações nos Testes de Adição Fraudulenta de Soro de Queijo

Gislene Bremer de Oliveira

Sob a Orientação da Professora
Rosa Helena Luchese

e Coorientação do Professor
Douglas McIntosh

Junho, 2013

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Tese submetida como requisito parcial para a obtenção do grau de **Doutor em Ciências** no Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Área de Concentração em Ciência de Alimentos

Seropédica, RJ
Junho de 2013

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APROVADA EM 04/06/2013

Rosa Helena Luchese, Ph.D. UFRRJ
(Orientadora)

Marcone Augusto Leal de Oliveira, Dr. UFJF
(Membro)

Marco Antonio Sloboda Cortez, Dr. UFF
(Membro)

José Francisco Pereira Martins, Ph.D. UFRRJ
(Membro)

Valéria Moura de Oliveira, Dra. UFRRJ
(Membro)

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AGRADECIMENTOS

A você que sempre acreditou
e que nos momentos mais difíceis
não ousou desistir.
Que fez mágicas diariamente
para transformar meus sonhos em
realidade e que, com amor, me apoia.
*A você, minha **mãe** querida,*
dedico este trabalho.

*"Tantas vezes pensamos ter chegado
Tantas vezes é preciso ir além."*

Fernando Pessoa

RESUMO

Fraude em leite por adição de soro é um problema recorrente no Brasil, enquanto a contaminação e deterioração do leite por bactérias psicrotróficas é um dos principais problemas enfrentados pela indústria de laticínios. Dentre estas bactérias, o gênero *Pseudomonas* tem sido destacado em diversos estudos microbiológicos como grupo predominante na microbiota do leite refrigerado. Mais recentemente, esta predominância tem sido confirmada através da aplicação de técnicas moleculares de identificação microbiana. Ainda, análises moleculares têm servido para levantar questões a respeito da precisão de algumas identificações fenotípicas realizadas anteriormente, e em relação ao papel de gêneros e espécies não detectados ou subestimados, e que podem ser encontrados como componentes principais da microbiota do leite refrigerado. Problemas relacionados à qualidade do leite e derivados devido à sua multiplicação e atividade enzimática são somados ao fato de que as suas proteases podem provocar resultados falso-positivos na detecção de fraude por adição de soro. Esta prática, comum em alguns países, inclusive o Brasil, é normalmente detectada através da análise do índice de CMP. Neste estudo, o total de 12 linhagens de bactérias psicrotróficas isoladas de leite e identificadas fenotipicamente em estudo anterior (ARCURI et al., 2008) foram reidentificadas usando métodos genotípicos baseados na análise da sequência (16S rRNA) que codifica a subunidade menor do DNA ribossomal. Esta técnica proporcionou identificação em nível de espécie para quatro linhagens recebidas como *Acinetobacter* spp. como sendo *A. guillouiae* (3) e *A. johnsonii* (1), além de identificar como *P. rhodesiae* uma linhagem recebida como *P. fluorescens*. Adicionalmente, três linhagens recebidas como *P. putida* foram reclassificadas como *P. psychrophila* e uma linhagem fenotipicamente identificada como *Aeromonas hydrophila* foi identificada como *A. punctata*. O crescimento dessas bactérias em temperatura de refrigeração foi avaliado através da inoculação no leite (*ca* 10^3 UFC mL^{-1}) e incubação em diferentes tempos/temperaturas (refrigeração), em acordo com delineamento fatorial (2^2) com três repetições no ponto central. Após cada período, a produção de CMP foi avaliada por cromatografia líquida com filtração em gel (HPLC-FG) e os resultados de um subgrupo de amostras foram comparados com os obtidos por teste imunocromatográfico rápido e por cromatografia líquida com ionização em eletrospray acoplada a espectrometria de massas em modo *tandem* (HPLC-ESI-MS/MS), cuja sensibilidade torna possível identificar resultados falso-positivos. A produção de CMP avaliada por HPLC-FG foi maximizada pela condição 10°C/4d para todas as linhagens, exceto para acinetobacters. Duas linhagens de *P. rhodesiae* apresentaram os maiores níveis, seguido por uma linhagem *A. hydrophila* (P07). Modelos lineares foram construídos para crescimento bacteriano, no entanto, para a produção de CMP 66.6% das amostras apresentaram tendência a comportamento quadrático. A análise por HPLC-ESI-MS/MS mostrou que a percentagem de pseudo CMP na condição 10°C/4d foi superior a 50% em

relação ao CMP verdadeiro em todas as amostras, exceto para a inoculada com *A. punctata* (P65). Apesar da temperatura (10°C) não ser permitida na legislação brasileira para armazenamento do leite, os dados demonstram o potencial dessas linhagens em gerar resultados falsos positivos (por HPLC-FG) para a adição fraudulenta de soro no leite.

ABSTRACT

Keywords: HPLC-GF, fraud, *Pseudomonas*

Fraud in milk by whey addition is a recurring issue in Brazil, while the contamination and spoilage of milk by psychrotrophic bacteria is one of the main problems faced by the dairy industry. Among these bacteria, the genus *Pseudomonas* has been highlighted in numerous microbiological studies as the predominant group in the microbiota of cold stored milk. Recently, this predominance has been confirmed via the application of molecular techniques for microbial identification. Yet, molecular analyses have served to raise questions regarding the accuracy of some earlier phenotypic identifications, and concerning the role of previously undetected and/or underestimated genera/species which may be found as major components of the microbiota of cold stored milk. Problems related to the quality of milk and dairy products due to the growth and enzymatic activities of psychrotrophs are compounded by the fact that the production of proteases by the bacteria may cause false-positive results in tests employed for detection of milk fraud by whey addition. This is a common practice in some countries, including Brazil, and usually detected by analyzing the CMP index. In the current study, a total of 12 strains of psychrotrophic bacteria isolated from milk and phenotypically identified in a previous study (ARCURI et al., 2008) were reidentified using genotypic methods based upon analysis of the sequence (16S rDNA) encoding the small subunit of ribossomal RNA. This approach provided identification to the species level for four strains received as *Acinetobacte*r spp. as *A. guillouiae* (3) and *A. johnsonii* (1), and identified as *Pseudomonas rhodesiae* an isolate received as *P. fluorescens*. In addition, three isolates received as *P. putida* were reclassified as *P. psychrophila* and an isolate phenotypically identified as *Aeromonas hydrophila* was identified as *A. punctata*. The growth of these bacteria at refrigeration temperatures was examined by inoculation of milk (ca 10^3 CFU mL^{-1}) with incubation at different times/temperatures (refrigeration), in accordance with a factorial design (2^2) with three repetitions at the central point. After each incubation period, the production of CMP was assessed by high performance liquid chromatography (HPLC-GF) and the results of a subgroup of samples were compared with those obtained by immunoassay and liquid chromatography coupled with electrospray ionization mass spectrometry in tandem mode (HPLC-ESI-MS/MS), which sensitivity makes possible the identification of false-positive results. The production of CMP, as assessed by HPLC-GF was maximal at 10°C/4d for all strains, with the exception of the acinetobacters. Two strains of *P. rhodesiae* showed the highest levels, followed by a strain of *A. hydrophila* (P07). Linear models were constructed for bacterial growth; however, for CMP production 66.6% of the samples showed a quadratic trend. The HPLC-ESI-MS/MS analysis showed that the percentage of pseudo CMP in some samples (condition 10°C/4d) was in excess of 50% compared to true CMP for all samples, except for the milk inoculated with an isolate identified as *A. punctata* (P65). Despite the fact that the temperature (10°C) is not permitted under Brazilian legislation for

milk storage, the data demonstrated the potential of these strains to generate false-positive results (via HPLC-GF) for the fraudulent addition of whey.

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1 INTRODUÇÃO GERAL

A refrigeração e a racionalização da coleta e o transporte do leite bovino no Brasil é, atualmente, regulamentada pelo Ministério da Agricultura, Pecuária e Abastecimento (MAPA) pela Instrução Normativa (IN) 62 (BRASIL, 2011)[1]. O objetivo é reduzir custos operacionais, principalmente os relacionados ao transporte, e promover melhorias na qualidade do leite. Esta regulamentação vem disseminando o uso de tanques de refrigeração e, entre outras determinações, o leite refrigerado no estabelecimento produtor imediatamente após a ordenha deve ser transportado para o estabelecimento processador em tanques isotérmicos, sendo previsto, também neste local, a permanência em temperatura de refrigeração. Somando-se estes períodos, é de se esperar que o leite permaneça refrigerado entre 24 e 120 h antes do seu processamento final.

Este processo é uma tendência mundial que beneficia toda a cadeia do leite, e a refrigeração imediatamente após a ordenha, apesar de diminuir a multiplicação de bactérias mesófilas que causam a acidificação do leite, favorece a microbiota psicrotrófica, que pode provocar diversas alterações tanto no leite como nos derivados.

Uma consequência direta das altas contaminações do leite por bactérias psicrotróficas é o fato de produzirem proteases termoestáveis até mesmo ao tratamento UHT, com capacidade para hidrolisar a κ-caseína no mesmo ponto de atuação da quimosina no processo de fabricação de queijos. A análise do peptídeo gerado pela quimosina, conhecido como caseinomacropeptídeo (CMP), por ser solúvel no soro, é utilizado como marcador de fraude por adição deste ao leite.

A adição fraudulenta de soro ao leite é financeiramente atrativa devido ao seu baixo ou nenhum custo, além da grande disponibilidade e alto custo envolvido no seu tratamento. Este tipo de fraude tem sido denunciada no Brasil. No entanto, como qualquer outra, a adição de soro ao leite é proibida pela legislação brasileira por reduzir o valor nutritivo deste alimento e diluir os teores de proteínas e gorduras. Caracteriza, ainda, prática abusiva que lesa os direitos do consumidor. No entanto, para uma grande diversidade de outros produtos como leites fermentados, achocolatados, confeitos, panificações e as denominadas bebidas lácteas, o soro é um ingrediente de uso comum.

Os Laboratórios Nacionais Agropecuários (LANAGROS) até março de 2010 pesquisavam a presença do CMP no leite exclusivamente através da cromatografia líquida de alta eficiência com filtração em gel (HPLC-FG). Com a publicação da IN 7 (BRASIL, 2010), eletroforese capilar e espectrometria de massas foram também incluídas na lista de técnicas passíveis de utilização para este tipo de determinação. Estas técnicas, apesar de mais específicas, portanto, menos sujeitas à interferência devido à ação de proteases produzidas por bactérias psicrotróficas, ainda não estão sendo utilizadas na rotina.

Dentre as bactérias psicrotróficas, o gênero *Pseudomonas* é relatado como o mais adaptado às condições atuais de estocagem do leite e, portanto, predominante na microbiota do leite refrigerado. Este gênero tem potencial proteolítico reconhecido, inclusive no que diz

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[1] BRASIL. Instrução Normativa Nº 62, de 26 de dezembro de 2011. Aprova o 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. Diário Oficial da União, 30.12.2011. 2011.

respeito à interferência nos testes de detecção de adição fraudulenta de soro no leite. No entanto, diversos outros micro-organismos psicrotróficos, também comuns na microbiota do leite cru refrigerado, ainda não foram estudados com referência ao seu potencial proteolítico e consequente capacidade de interferência nos teste.

Considerando-se a grande importância do leite na dieta, a necessidade de assegurar a sua qualidade, combater fraudes e proteger o consumidor, torna-se necessário a realização de pesquisas capazes de esclarecer esta interferência e, consequentemente, sustentar a correta avaliação. Desta forma, espera-se que medidas penais cabíveis possam ser aplicadas ao fraudador.

Organizada na forma de capítulos, esta tese visa discutir os seguintes tópicos: a) o problema de fraude no leite no Brasil, tendo em vista um episódio de grande repercussão ocorrido em 2007; b) a presença de bactérias psicrotróficas no leite refrigerado e mudanças de paradigma apresentados devido ao surgimento e avanço métodos de identificação genotípica; c) os resultados de um estudo comparativo de métodos de identificação dessas linhagens e, por último d) apresentar resultados de um estudo comparativo de métodos utilizados na detecção de CMP.

2 JUSTIFICATIVA E OBJETIVOS

2.1 Justificativa

É necessário conhecer o potencial de crescimento e proteolítico de um maior número de linhagens e gêneros de bactérias psicrotróficas comumente isoladas de leites contidos em tanques de refrigeração de leite no novo cenário apresentado pela produção de leite no Brasil. Estas informações poderão corroborar no estabelecimento de critérios de orientação ao setor público e privado com relação ao controle de qualidade do leite. Da mesma forma, a avaliação da capacidade destas bactérias em produzir CMP poderá ser útil para o aperfeiçoamento das metodologias utilizadas pelo MAPA.

2.2 Objetivos

2.2.1 Geral

Caracterizar a atividade proteolítica da microbiota psicrotrófica autóctone do leite cru produzido no Brasil e mantido nas temperaturas de refrigeração preconizadas pela IN 62 e abusiva a 10ºC.

2.2.2 Específicos

a) Realizar a identificação genotípica das diferentes espécies/linhagens de bactérias psicrotróficas isoladas de leite;

b) Avaliar a atividade proteolítica dessas espécies/linhagens qualitativamente por plaqueamento em ágar leite e quantitativamente pelo método colorimétrico da azocaseína;

c) Testar todas as bactérias em cultura axênica inoculando-as em leite tratado termicamente em quantidade suficiente para obter populações de ca 1 x 10^3 UFC mL^{-1} e incubando o leite contaminado em diferentes binômios tempo/temperatura;

d) Determinar um modelo para o crescimento bacteriano e produção de CMP analisado através da HPLC-GF;

e) Analisar a produção de CMP de amostras selecionadas através de teste imunocromatográfico e comparar os resultados com os obtidos através da HPLC-GF;

f) Analisar a produção de CMP de amostras selecionadas através de HPLC-ESI/MS/MS;

g) Tratar as amostras termicamente no final do período de incubação estabelecidos na etapa (c) e incubar uma alíquota a 30°C/120 dias;

h) Obter o perfil zimográfico das proteases produzidas por linhagens selecionadas.

CAPÍTULO I

MILK FRAUD IN BRAZIL FROM THE 2007 SCANDAL: UNDERSTANDING THE DRAWBACKS FOR BETTER PLANNING

ABSTRACT

The defraudment of food is, regrettably, an old practice. In 2007, a scheme of milk adulteration in Brazil was denounced and exposed to the media with deep repercussion to the industry. The consequence was lesser milk consumption and reduced payment to producers of the region, coupled with serious damage to the reputation of the entire sector. Most reports released at the time were non-consistent for some of the adulterants mentioned. Similar frauds occurred before and after this event. Questions remain about investments in fraud control and prevention. The aim of this article is to discuss food defrauding, taking the 2007 fluid milk scandal as a reference and pointing areas where investments are most needed.

Keywords: milk adulteration, food control, quality insurance

RESUMO

Fraude em alimentos é, infelizmente, uma prática antiga. Em 2007, um esquema de adulteração de leite no Brasil foi denunciado e exposto à mídia com profunda repercussão para a indústria. A consequência foi menor consumo de leite e menor preço pago aos produtores da região, juntamente com graves danos para a reputação de todo o setor. A maioria dos relatórios divulgados na época não foi consistente para alguns dos adulterantes mencionados. Fraudes similares ocorreram antes e depois deste evento. Ainda restam dúvidas sobre os investimentos em controle e prevenção de fraudes. O objetivo deste artigo é discutir fraude em alimentos, tendo o escândalo do leite fluido de 2007 como referência, apontando as áreas onde os investimentos são mais necessários.

Palavras-chave: adulteração do leite, controle em alimentos, garantia da qualidade

1 INTRODUCTION

"Nobody likes to be cheated." With these words Hermans (2012) commences a text on food fraud. About the same subject wrote Shears (2010): "a current issue but an old problem". While Perks (2007) calls it as "the perfect crime", for Shears (2010) it is "a big business". These details draw attention to the problem that today, due to markets globalization, can reach large proportions, and that needs to be striven.

The risks of acquiring food adulterated by deception are many and can range from simple financial loss to nutritional deficits, allergic reactions with varying degrees of severity that can even lead to death.

Worldwide, the most common milk adulteration is the addition of water, though nowadays most dairy plants, milk collection facilities and laboratories are fully equipped with cryoscope and/or other instruments to detect this fraud. However, the addition of sweet whey from the cheese production as an adulterant remain and is difficulty or expensive to detect.

Although milk analysis for inspection purposes is not a new subject, in Brazil these actions became more visible to the public in 2007, when the so-called "milk fraud scandal" happened (MAPA, 2007a). A former employee accused of deception one of the three companies involved, thus, a large scheme of adulteration was unveiled in Minas Gerais state by a combined task-force comprising the Federal Police and officers of the Ministry of Agriculture, Livestock and Supply (MAPA). This operation was called "Operação Ouro Branco" ("White Gold Operation"). Unfortunately, in this episode, the broad status of the term fraud was given to which should be properly classified "economically motivated adulteration", according to Food and Drug Administration (FDA, 2009). The accusation was that the company added hydrogen peroxide, sodium hydroxide and water into milk. The media and, therefore, the public, had wide access to the ongoing investigation. As a consequence there was a significant decrease in milk consumption and fall of prices, penalizing mostly the milk farmers.

Because of milk upmost importance in the diet, as essential for children growth and development, as well as for contributing to maintain the elderly in healthy conditions, this fact caused great popular concern among consumers.

Thereby, in the following year, issues relating to milk inspection led the ranking of phone calls to MAPA ombudsman office, which resulted in broader spread of information (MAPA, 2009a).

The major difficulty in counteracting this practice is that consumers not always perceive, exactly, when they are victims, because the sound and the adulterated products may have similar flavor and appearance and costing almost the same. Therefore, apparently, it does not necessarily represent a damage except the financial loss (PERKS, 2007; SHEARS, 2010). This situation also makes difficult to predict how widespread the problem is. It is estimated that in the UK alone, this practice may reach 10% of the food produced, which means the loss of hundreds of millions of pounds (SHEARS, 2010).

Unfortunately, the fraud occurred in Brazil in 2007 was not an isolated case. The aim of this paper is to discuss about elements and concepts of food fraud and, and referring to fluid milk and "milk fraud scandal", pointing out facts similar less publicly explored. Finally, we discuss the difficulties to unveil and collect evidence to take the fraudster to criminal Court.

2 LITERATURE REVIEW

2.1 A Hint of History

The protection of wholesomeness of a nation's food supply in a nation has long been a governmental task. Early reports of food fraud came from Ancient Rome, but, nowadays, it has become more sophisticated (RANDELL, 1995; DOWNEY, 1996; KAROUI; DE BAERDEMAEKER, 2007; WILSON, 2008; SOUZA et al., 2011; SPINK; MOYER, 2011).

A pioneer in this topic was Friedrich Accum, chemist born in Germany but living in London from 1793 to 1821 (COLE, 1951). In 1820 he published a book entitled "A treatise on adulterations of food, and culinary poisons, and methods of detecting them". The preface says:

"*To such perfection of ingenuity has the system of counterfeiting and adulterating various commodities of life arrived in this country, that spurious articles are every where to be found in the market, made up so skillfully, as to elude the discrimination of the most experienced judges… But of all of possible nefarious traffic and deception practiced by mercenary dealers, that of adulterating the articles intended for human food with ingredients deleterious to health, is the most criminal, and, in the mind of every honest man, must excite feelings of regret and disgust… The eager and insatiable thirst for gain, is proof against prohibitions and penalties; and possible sacrifice of fellow-creatures life is a secondary consideration among unprincipled dealers*" (ACCUM, 1820).

In this book, Accum deals with fraud in various types of foods such as wine, beer and other alcoholic beverages, as well as bread, coffee, cheese, pepper, pickles, olive oil, among others. The allegations of Accum ranged from the simple addition of peas into coffee to lead contamination of olive oil from containers used in processing.

Accum exposed names of some fraudsters already prosecuted and convicted, and received anonymous threats that he mentioned in a second edition of the book, published in that same year (COLE, 1951).

2.2 Food Fraud Definitions

Food fraud is a broad term comprising the deliberate and intentional substitution, addition, tampering, or misrepresentation of food, food ingredients, or food packaging; or false or misleading statements made about a product, for economic gain (SPINK; MOYER, 2011).

Another definition proposed by the Food Standards Agency was: "*deliberately placing on the market, for financial gain, foods which are falsely described or otherwise intended to deceive the consumer*" (FSA, 2007).

In 2009, the FDA proposed that economically motivated adulteration (EMA) is: "*fraudulent, intentional substitution or addition of a substance in a product for the purpose of increasing the apparent value of the product or reducing the cost of its production, i.e., for economic gain*" (FDA, 2009).

Hermans (2012) discussed the idea that financial gain is automatically implied in the word "fraud". This author also enforces that two forms of fraud can be distinguished: on in which results of evaluated parameters are outside the regulatory limits; while another, the results, although within regulatory margins, deliberately mislead the consumer.

2.3 Fraud in Foods of Animal Origin

Unlike few years ago, today's food consumer is concerned about food attributes such as origin, method of production, e. g., whether it is genetically modified, organic or if the animal was raised free or confined, as well as ethical issues involving animal welfare, fair trade and sustainability (PERKS, 2007).

These concerns may give the idea those coarse situations such as added water, starch, additives and/or addition of extraneous proteins into meat products, as well as addition of commercial glucose or syrup into honey were problems of the past. However, surely this is not the reality.

Milk is an expensive raw material (DE LA FUENTE; JUÁREZ, 2005). Thereby, the quest for financial gain is in itself considered a reason for its fraud. The milking conditions, the time it is normally stored before processing and for being a favorable medium for bacterial growth help to explain its vulnerability.

Indeed, wide collection and analysis of data was reported by Moore et al. (2012). These authors collected both academic and media data of economically motivated adulteration and food fraud in between 1980-2010. Based on academic data they concluded that from the top foods analyzed, milk was second (14%) only to oils in the rank (24%) among those involved in the largest number of cases.

Fraud in milk is usually separated into two types: a) sanitary: refers procedures to mask deficiencies of the product, by adding extraneous substances which, potentially, can cause health problems; b) economic: refers to adding innocuous substances to increase the volume of milk, without harm to health (ABLV, 2007a).

Nowadays, these frauds have been practiced together, since chemicals capable of acting, e. g., as restorative density may be used to mask the addition of water or whey, commonly used in economic frauds. Additionally, neutralizing agents and preservatives act so as to restore and preserve the quality of milk.

2.4 Milk Fraud in Brazil

The Brazilian Regulation for Industrial and Sanitary Inspection of Animal Products (RIISPOA), in the article 543, considers defrauded, adulterated or counterfeited, among others, the milk added of water or substances or any preservative extraneous matter to its composition (BRASIL, 1952). An exception to this rule is found in the Technical Regulation of Identity and Quality of UHT Milk, which approves the use of sodium citrate as stabilizer. Citrate and other approved additives, such as mono-, di- and tri-sodium phosphate, separate or combined, can be also used for the same purpose (BRASIL, 1997).

The Brazilian Penal Code states penalty of one to two years imprisonment plus fine to whom "corrupt, tamper, alter or counterfeit substances or food product, making it harmful to health or reducing its nutritional value". These punishments can be applied after proven guilty in "who manufactures, sells, offers for sale, has in storage to sell or in any way distributes or delivers for consumption adulterated or counterfeit food product" (BRASIL, 1998).

Official information about situations involving milk fraud in Brazil are available on the MAPA website, giving ideas about how this problem has been occurring in this country in recent years.

In March 2003, the Department of Inspection of Animal Products (DIPOA) of MAPA closed a dairy company in Recife, northeastern state of Pernambuco, for adulterating milk

powder. The company added maltodextrin and whey powder into milk. At the time, 9.6 t of milk powder, 3.75 t of maltodextrin and 1.7 t of whey powder were seized in the company warehouse. Analysis of milk samples collected prior to the date of sanction resulted in 21.8% and 20.7% of added whey, and the samples collected on the day of the closure had 23.7%. This company was milk powder supplier to school meals in 15 towns and cities of that state (MAPA, 2003b).

An action of milk fraud task-force of MAPA, in April 2003, detected the whey addition into milk in 32 out of 74 (43%) of industries sampled over a four months in 13 states. Almost 35% of the 341 samples tested positive in laboratory. This action seized 18.3 tons of UHT milk and 1.9 tons of defrauded milk powder (MAPA, 2003a).

In March 2004, the MAPA press office reported that inspection was intensified to counteract fraud in milk production and trading, jointly with the Federal Police. The director of DIPOA stated that the most common fraud applied to fluid milk was water and/or whey addition (MAPA, 2004).

From January to August of 2007, 355 milk samples were tested by the MAPA official laboratory (LANAGRO) and 59 of them were suspected of fraud by whey addition (MAPA, 2007c).

Also in 2007, MAPA has announced changes in the inspection regime, aiming to increase the responsibility of the industries regarding their products. The requirement for a permanent governmental inspector in the companies was dropped (MAPA, 2007b).

In May 2008, a joint task-force of Federal Police and MAPA inspectors dismantled a criminal organization active in the states of Paraíba, Pernambuco, Ceará, Bahia and Santa Catarina, that was adulterating milk powder by adding whey and other dairy ingredients. This operation was known as "Operação Lactose" (Lactose Operation) (MAPA, 2008).

Fraud evidences led to the seizure of more than 100.000 L of milk in São Paulo, Mato Grosso do Sul and Paraná states in 2009. These evidences emerged from the documental analysis of reception of raw materials, production and trading of finished products, as well as the inventory control of ingredients and additives used in the manufacturing processes. Out of the total tested and seized, 14.000 L of raw milk had non-permitted substances and 36.659 L of refrigerated raw milk with quality parameters out of legal limits. The remaining 60.000 L of UHT milk, ready for distribution, were seized for suspected water addition. The companies were fined and subject to special close monitoring scheme (MAPA, 2009d).

In response to this action, the MAPA explained that from the second half of 2007 to May 2009, the DIPOA had performed more than a thousand laboratorial tests for fraud detection. As a result, 40 companies were subject to special monitoring regime, one company lost its license in the Federal Inspection Service (SIF), and two others did not fulfill the requirements and determinations of DIPOA, and had the manufacturing activities suspended. In addition, the MAPA stated that over that same period, more than 500 administrative proceedings were opened against the companies on the matter (MAPA, 2009c).

Companies subject to special close inspection regime were allowed to trade their products after daily sampling and testing, showing results within acceptable values over a two weeks period. Furthermore, the companies had to implement a plan of corrective actions as response to non-compliances found by the auditors (MAPA, 2009b).

Even in 2009, the MAPA reported that the number of milk samples tested had increased by 128% in 2009, to a total of 2934 samples. Out of this total, 28 samples had indication of fraud, and the manufacturers were subject to penalties (MAPA, 2010).

In 2011, other milk fraud have been investigated and/or proved, as the one practiced by milk farmers in Casca - RS, and others arising from "Operação Ouro Branco II", launched by the Federal Police in Leopoldina and Campo Belo - MG . However, there were no official publications of these occurrences by MAPA.

The General Controller of the Union (CGU) and the Federal Police in a 2012 joint operation called "Amaltéia" found that water and other chemicals were added to milk by participants of the official governmental program "Leite da Paraíba" among several others administrative fraud (CGU, 2012).

More recently, added water concealed by the fertilizer urea was unveiled in Rio Grande do Sul state. The determination was possible due to the presence of formaldehyde, also a constituent of the fertilizer. This joint operation of the MAPA and the RS's Public Prosecutor's office was called "Leite Compen$ado" ("Compensated Milk") and, according to them, the fraud was practiced independently by milk transporters (MAPA, 2012).

2.5 Milk Fraud Detection in Brazil, and the Reported Case of 2007

Traditional wet analyses strategies have been used to test for adulteration and to ensure the quality of food products. In this way, it is necessary to set the amount of one or more markers compounds in a suspected sample and compare the value obtained in the procedure with equivalent food of known origin (DOWNEY, 1996).

Brazilian regulation states official methods for the detection of foreign substances in milk which are included the IN 68 (BRASIL, 2006a). These are, generally, fast qualitative methods for determination of acidity neutralizing agents, some density restoratives compounds (e.g., starch and sodium chloride), and preservatives (such as hypochlorite, hydrogen peroxide and formaldehyde). The same IN includes routine tests such as density and freezing point to detect water addition in milk, and also mentions alkalinity in ash as an indicator of the use of neutralizing acidity.

The milk samples seized and tested at "Operação Ouro Branco" were reported to have high levels of alkalinity in ash associated with the low acidity, which the National Health Surveillance Agency (ANVISA) considered to be indicative of fraud, but the presence of NaOH was not confirmed, as previously reported (ANVISA, 2007).

A problem faced in this type of analysis is the presence of sodium citrate in UHT milk, an allowed additive which increases the content of alkaline substances in the milk. For this reason, this test can be applied solely to raw milk and pasteurized milk (MILAGRES, 2008).

With respect to hydrogen peroxide, the ANVISA has recognized that this is an additive referred to as technology adjuvant in food, especially as reducer of microbial load, but its use in milk is not approved in Brazil. Based on FAO information data (FAO, 1967), they also reported that excess hydrogen peroxide is destroyed, and that toxicological considerations should account the residual hydrogen peroxide, but the potential interference with the nutritional value or the formation of substances toxic in the treated food.

The IN 68 presents two alternatives for the research of adding whey to milk: a) detection of free sialic acid via spectrophotometric method. Sialic acid is a component of the caseinmacropeptide molecule (CMP), a marker used worldwide for this purpose (FURTADO et al., 2011); b) detecting the CMP itself via HPLC. The acceptable level of CMP in milk, as well as its proper destination in case this level is exceeded is regulated by IN 69 (BRASIL, 2006b).

However, the detection of CMP is hampered by the action of enzymes produced by psychrotrophic bacteria, common in the refrigerated milk microbiota, which can lead to false-positive results (RECIO et al., 1996; ÚRBAN et al., 1998; RECIO et al., 2000; BREMER et al., 2008; OLIVEIRA, 2009). For this reason, the reports of the tests conducted by the Institute of Criminology in 2007, showed to be non-conclusive for the presence of whey in milk (ABLV, 2007b).

Subsequently, refinement in the analysis of milk CMP was published on IN 7 (BRASIL, 2010), where capillary electrophoresis and mass spectrometry were also included and the method of sialic acid was deleted from the list of techniques that could be used for this type of determination. According to this IN the new techniques "allow differentiation between CMP from cheese whey and similar peptides produced by bacterial proteolysis of milk". However, even though implanted, these techniques have not yet been implemented.

2.6 Tendencies in Milk Fraud Detection

Nowadays food fraud or adulteration tends to become more subtle and sophisticated than before making more difficult its detection using traditional physical and chemistry methodologies. The need for results to be get in short time, the exemption of prior sample preparation and being non-destructive tests lead to feasibility of application of spectrum fingerprinting methodologies (e.g. nuclear magnetic resonance, ultraviolet, mass spectrometry, so-called "four spectrum" in organic analyses) in programs of milk quality control (ZHANG et al., 2011).

Recently, the International Dairy Federation (IDF) confirmed to be working in the development of an untargeted infrared (IR) based approach to prevent milk adulteration (ASTLEY, 2012). Although the method can be also useful for known adulterants, the great advantage of this method is that also makes possible to detect an endless number of contaminants, as far as standards to normal milk are established.

In Brazil, Fourier transform infrared spectroscopy (FTIR) was used by Cassoli et al. (2011) to identify raw milk samples adulterated with sodium bicarbonate, sodium citrate and cheese whey. At first, these authors built a reference spectrum from 800 representative samples. The results for sodium bicarbonate and sodium citrate were within an acceptable range. However, the method was not sensitive enough to detect even high additions of whey (20%). Changes on milk composition that were significative in whey-added samples could not be differentiated from the reference spectrum.

Mid-infrared microspectroscopy (MIR-microspectroscopy) coupled to multivariate analysis was also tested to detect milk adulterators such as whey, hydrogen peroxide, synthetic urine, urea and synthetic milk. The method, according to authors, was suitable to detect almost all those substances, but the difficulties to reveal added-whey remained, with the highest standard error of prediction compared to the other adulterants used in the test-model (SANTOS; PEREIRA-FILHO; RODRIGUEZ-SAONA, 2013)

The low accuracy of spectrum fingerprinting methods leading to non-precise results and the need for a software to analyze the complex spectrum have been pointed as drawbacks of these methodologies (ZHANG et al., 2011).

2.7 Food Fraud Control and Prevention

The activity of fraud food is dynamic, and the fraudster is usually compromised in developing new and more subtle and sophisticated forms of fraud. These may include new contaminants, some unconventional, with potential risk greater than those already known to public health. The fraud control must respond to this pressure with equal dynamism seeking not only monitor but anticipate the fraudster.

The current intervention systems are designed to search for specific contaminants. However, the number of potential contaminants tends to infinity (SPINK; MOYER, 2011), which generates continuing demand for novel methods, rapid, highly sensitive and unquestionable for assessing quality and tamper detection in milk (KAROUI; DE BAERDEMAEKER, 2007; GARCIA et al., 2012). Specifically, in Brazil, prioritization and optimization of these activities are required in all states of the federation (SOUZA et al., 2011).

The low reliability and accuracy of the methodology used, if questioned in the Court, may give a chance to fraudsters to run-free from any conviction (GARCIA et al., 2012). Simpkins and Harrison (1995) state that the Court is the final test of the ability of the expert, where supposedly guilty parties may cast doubt on the reliability of the evidence presented.

The prevention of food fraud is a complex and interdisciplinary subject relying on support from the supply chain, manufacturing, packaging, procurement, corporate security, food law, public health, consumer behavior, social anthropology and criminology (SPINK; MOYER, 2010).

Encouraged by industries and governmental agencies, researchers of the Michigan State University created the Anti-Counterfeiting and Product Protection Program. It was organized to be "independent, interdisciplinary evidence-based hub, offering research, educational programs, information, and partnership opportunities designed to assist in protecting brands and products of all industries worldwide". The goal of such initiative is to change the focus of the response from intervention to prevention through understanding. It became clear that not only the experimental development of new detection methods and technologies, but understanding the basic opportunity of fraud is important. This involves understanding the types of fraudsters and fraud across the behavioral sciences, criminology included. For over 30 years, the field of criminology has focused on changing the environment in which a crime occurs and propose this strategy as a way to reduce its attractiveness. According to experts, these concepts are directly applicable to food fraud (SPINK; MOYER, 2010).

3 CONCLUSION

It is adamant opinion that more accurate and sensitive methods for detecting fraud in food are, nowadays, more needed than ever. The low reliability of the methods used in the case of 2007 milk fraud scandal in Brazil exposed technical shortcomings of the official laboratories responsible for inspection, leading to results that may be challenged in Court. Also exposed was a lenient and sometimes negligent behavior of the entire milk industry. The event left open questions about the extent of the practice of defrauding milk with serious long-term damages to the image of the entire milk market.

How far the conclusions taken at the time, that fed Court proceedings, could have been obtained by the then current dynamics of inspection and quality assurance laboratory procedures if a former employee had not made the complaint? From that time, how much has been invested in the development, deployment and implementation of new and improved methods and techniques? How much has been invested in new and improved equipment and other laboratory facilities? How has been the investment into improving and qualifying personnel? Are the inspection and quality assurance strategies being reassessed on a periodical basis?

The strategies of prevention fraud had a positive gain when the requirement for a permanent in-factory official inspector was dropped off making the system less prone to corruptive actions. However this should come coupled with strategies of investment in intelligent systems of creating truly qualified professionals and protocols to prevent such practices.

Most industries and producers are serious and honest in their practice, but the lack or lesser control against fraudster can dramatically reduce their competitiveness with strong potential do damage the entire industry.

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CAPÍTULO II

PSYCHROTROPHIC BACTERIA IN MILK: HOW MUCH DON'T WE KNOW?

ABSTRACT

The occurrence of psychrotrophic bacteria in raw milk has been studied worldwide owing to the difficulties in control their growth during cold storage and the consequent negative effects upon fluid milk or dairy products. Among the psychrotrophic bacteria, members of the genus *Pseudomonas*, represented primarily by the *P. fluorescens*, have been highlighted as the cause of numerous defects in dairy products and, consequently, economical losses for the dairy industry. In light of its perceived predominance, this species has frequently been chosen as a model organism with which to assess the effects of psychrotrophic bacteria upon milk. However, recent findings, derived from the application of molecular biology based techniques, have highlighted a number of deficiencies in our knowledge of the biology of milk associated psychrotrophic. In common with other areas of microbiology, the use of culture independent approaches to evaluate microbial population dynamics in milk and dairy products has demonstrated that the spectrum of species involved in low temperature milk spoilage is far wider than had been estimated via traditional culture based studies. Furthermore, it has been revealed that microbe to microbe communication, via mechanisms such as quorum sensing, plays a significant role in determining both which, and to what extent, different groups of microbes develop during cold storage of milk, and such cross-talk may also direct the expression of enzymatic activities associated with post-storage defects in dairy products e.g. cheese. Data from molecular identification methods i.e. genotypic identification, have also exposed errors in the classification of members of the genus *Pseudomonas* isolated from cold stored milk, and have stimulated a reevaluation of the presumed status of *P. fluorescens* as the predominant milk associated psychrotroph species. This chapter presents a succinct review of data from recent studies which contest existing theories in relation to the microbiology of cold stored raw milk.

Keywords: microbiology, *Pseudomonas*, dairy products

RESUMO

A ocorrência de bactérias psicrotróficas no leite cru tem sido estudada em todo o mundo, devido às dificuldades no controle de seu crescimento durante o armazenamento refrigerado do leite e os consequentes efeitos negativos sobre o leite e produtos lácteos. Entre elas, membros do gênero *Pseudomonas*, representados principalmente pela *P. fluorescens*, são apontados como causa de inúmeros defeitos em produtos lácteos e, consequentemente, perdas econômicas para a indústria de laticínios. Devido a sua conhecida predominância, esta espécie tem sido frequentemente utilizada como micro-organismo modelo em estudos de avaliação dos efeitos de bactérias psicrotróficas sobre leite. No entanto, descobertas recentes, derivadas da aplicação de técnicas baseadas em biologia molecular, têm revelado deficiências do nosso conhecimento sobre a biologia do leite associado com psicrotróficos. Em comum com outras áreas da microbiologia, o uso de métodos cultura independentes para avaliar a dinâmica da população microbiana em leite e derivados tem demonstrado que o espectro de espécies

envolvidas na deterioração do leite armazenado em baixa temperatura é muito maior do que havia sido estimado por estudos baseados em métodos tradicionais de cultura. Além disso, tem sido revelado que comunicação entre bactérias através de mecanismos como "quorum sensing" desempenha um papel significativo na determinação do como e em qual extensão diferentes grupos de micro-organismos se desenvolvem durante o armazenamento refrigerado do leite, podendo tal comunicação também direcionar a expressão das atividades enzimáticas associadas com defeitos de pós-armazenamento em produtos lácteos, por exemplo, queijos. Dados de métodos de identificação molecular, ou seja, de identificação genotípica, também tem exposto erros na classificação de membros do gênero *Pseudomonas* isolados de leite referigerado, e tem estimulado a reavaliação do status de predominância de *P. fluorescens* no leite associado com espécies psicrotróficas. Este capítulo apresenta uma sucinta revisão dos dados de estudos recentes que contestam as teorias existentes em relação à microbiologia do leite cru refrigerado.

Palavras-chave: microbiologia, *Pseudomonas*, produtos lácteos

1 INTRODUCTION

Psychrotrophics can be defined as microorganisms capable of producing visible growth (colonies or turbidity) within 7 to 10 days at temperatures equal to or less than 7°C, regardless of their optimum growth temperature (CROMIE, 1992; CHAMPAGNE et al., 1994). The ability to grow at low temperatures makes this group of microbes especially significant with regard to food spoilage and safety, given that the storage of many foods at cold temperatures is a routine practice during production, transportation and processing (RUSSELL, 2002; BEALES, 2006).

Raw milk provides a physicochemical environment favorable to the multiplication of a broad spectrum of microorganisms including a range of psychrotrophic bacterial species, predominantly members of the genus *Pseudomonas* (SØRHAUG; STEPANIAK, 1997; MCPHEE; GRIFFITHS, 2011), which contaminate the milk during collection and/or processing (MARCHAND et al., 2012).

Although pasteurization of raw milk decreases the microbial load, the efficiency of the process and the resulting quality of dairy products are directly influenced by the microbiological quality of the raw milk (NÖRNBERG et al., 2010).

Rigorous hygiene employed to reduce the possibility of exogenous contamination, coupled with low storage temperatures to control the growth of mesophilic organisms are essential components of the microbial control strategies employed with raw fluid milk (BARBANO; MA; SANTOS, 2006).

The presence and subsequent replication of populations of psychrotrophs may also lead to spoilage of the milk (BEALES, 2006). In light of the fact that the economic impact of this group of microbes upon the global dairy industry is substantial (RAATS et al., 2011), psychrotrophic bacteria have been, and continue to be studied extensively with the principal objective of developing effective control measures and establishing regulations to ensure the quality and safety of milk and dairy products (MCPHEE; GRIFFITHS, 2011).

Despite the existence of an extensive body of published data, it would be fair to state that numerous gaps exist in relation to our understanding of the biology of the psychrotrophic bacteria of importance for the dairy industry. In this context, the continued development of molecular tools for bacterial identification and their application to the analysis of microbial population structure and ecology in milk and dairy products has revealed the presence of psychrotrophic bacteria not detected by the use of traditional culture based approaches (MARCHAND et al., 2009b; RAATS et al., 2011; ALMEIDA; ARAUJO, 2013). Similarly, molecular methods have highlighted discrepancies in relation to the identification of the psychrotrophic species associated with spoilage of cold stored milk, particularly with regard to the genus *Pseudomonas* (MCPHEE; GRIFFITHS, 2011).

The majority of studies on milk associated psychrotrophs have focused upon individual isolates grown as planktonic cultures (readily cultivable). However, there is an increasing realization that such approaches overlook potential interactions and cross-talk between different species of psychrotrophic bacteria present within biofilms that can develop in milk storage and processing environments and which may exert an influence on milk quality and safety (CLETO et al., 2012; MARCHAND et al., 2012).

The current mini-review sought to collate and evaluate the findings of recent studies on psychrotrophic bacteria of importance to the dairy industry and to demonstrate that the

activities of psychrotrophic bacteria in milk are more extensive and more complex than was previously thought.

2 LITERATURE REVIEW

2.1 Biochemical Basis for Psychrotrophic Growth

Temperature influences bacterial growth rates by affecting the conformation of cellular macromolecules and other constituents, determining the rates of the hundreds of intracellular enzymatic reactions that are crucial for viability (RUSSELL et al., 1990; FONSECA; MORENO; ROJO, 2011). Hence, adaptation of the cell to low temperatures requires enzymes which are active at low temperatures (CHATTOPADHYAY, 2006), as well as the production of cold shock proteins (CSPs) and cold acclimation proteins (Caps) (BERGER et al., 1996).

Transcriptomic analysis of *Pseudomonas putida* strain KT2440 revealed that, in comparison to cells grown at 30°C, the expression of at least 266 genes (some 5% of the genome) was modified during low temperature growth. Several changes seemed directed towards neutralizing problems created by low temperature, such as increased protein misfolding, the increased stability of DNA/RNA secondary structures, reduced membrane fluidity and reduced growth rate (FONSECA; MORENO; ROJO, 2011).

Under low temperature growth conditions, psychrotrophic bacteria synthesize phospholipids and neutral lipids containing increased proportions of unsaturated fatty acids, leading to a reduction in the melting point of the lipids which serves to maintain their fluidity, thus permitting the functionality, solute transport, secretion of extracellular enzymes and fluidity of the membrane (JAY, 2005; BEALES, 2006).

2.2 Psychrotrophic Bacteria in Milk

Refrigeration, either alone or in combination with other methods such as the addition of preservatives, is the most commonly used means of preserving food, including milk and dairy products (BEALES, 2006).

The current trend in the dairy industries is to reduce the frequency of milk collection, thus the refrigerated storage of milk has been lengthened (FROHBIETER et al., 2005) from two to five days prior to heat treatment (O'BRIEN; GUINEE, 2011). This practice been stimulated in part by desire for the 5-day working week and also in response to decreased milk supply at certain times of the year (MCPHEE; GRIFFITHS, 2011).

The procedure of cooling and subsequent refrigerated storage of raw milk will effectively control the development of populations of mesophilic spoilage organisms, but at the same time it serves to provide a selective pressure for the growth of psychrotrophic bacteria (BARBANO; MA; SANTOS, 2006; DE JONGHE et al., 2011; SAMARŽIJA; ZAMBERLIN; POGAČIĆ, 2012).

The microbiota of raw milk can vary greatly both in terms of microbial load and species diversity (MUNSCH-ALATOSSAVA; ALATOSSAVA, 2006; MCPHEE; GRIFFITHS, 2011). In this context, the health status of the cattle, the nature of their feed and the storage conditions of raw milk are key factors which determine the composition and size of the microbial population (MARCHAND et al., 2009a).

According to Sørhaug and Stepaniak (1997) and Champagne et al. (1994) psychrotrophic bacteria in milk are represented predominantly by Gram-negative genera such as *Pseudomonas*, *Achromobacter*, *Aeromonas*, *Serratia*, *Alcaligenes*, *Chromobacterium* and

Flavobacterium spp. and, in much lower numbers, by Gram-positive genera including *Arthrobacter*, *Bacillus*, *Listeria*, *Micrococcus*, *Staphylococcus*, *Clostridium*, *Corynebacterium*, *Streptococcus*, *Lactobacillus* and *Microbacterium* spp.

Interestingly, milk freshly drawn from the udder may often not contain detectable populations of cultivable psychrotrophic bacteria. Indeed, it has been reported that they frequently account for less than 10% of the initial raw milk microbiota (COUSIN; BRAMLEY, 1981; MCPHEE; GRIFFITHS, 2011). However, they will develop over time in virtually all cold stored raw milk, a feature which make the normal refrigerated storage life usually less than 5 d (CHEN; DANIEL; COOLBEAR, 2003; MA; BARBANO; SANTOS, 2003; RAATS et al., 2011).

The average psychrotrophic aerobic bacterial count of milk silos at several dairies in southwest Scotland was recorded as being 1.3 x 10^5 CFU mL^{-1}. The majority of bacteria present were Pseudomonads (70.2%), but Enterobacteriaceae (7.7%), Gram-positive bacteria (6.9%), and other Gram-negative, rod-shaped organisms were also isolated. When the milk was stored for a further 48 h at 6°C, the psychrotrophic counts increased by two log cycles to 1.3 x 10^7 CFU mL^{-1} (MCPHEE; GRIFFITHS, 2011).

In addition to growing at low temperatures, a number of the psychrotrophic bacterial species, primarily pseudomonads, found in raw milk produce heat-stable proteases (LIU; WANG; GRIFFITHS, 2007) and lipases (DIECKELMANN; JOHNSON; BEACHAM, 1998), generally during the late log or early stationary growth phases, when cell density is high. Many psychrotrophs derived enzymes produced in bulk tank raw milk during refrigerated storage are heat stable and retain significant activity after pasteurization (72-75°C/15-20s) and even UHT treatment (13-150°C/2-4s), and may subsequently degrade proteins and fats present in processed products, resulting in reduced shelf life despite the absence of viable bacteria (BOOR et al., 1998; DUNSTALL et al., 2005; BARBANO; MA; SANTOS, 2006; DE JONGHE et al., 2011).

Reduction of cheese yield and tainting are the two most frequently reported negative effects upon cheese milk that are caused by psychrotrophic derived enzymes (MCPHEE; GRIFFITHS, 2011). Less frequently reported effects include alteration of starter activity and growth and rennet coagulation time (DATTA; DEETH, 2001; MANKAI et al., 2012).

Reduced yields in cheese production occur mainly due to the fact that casein degradation products (peptides and amino acids), which are soluble, may be lost into the whey instead of forming part of the curd (MCPHEE; GRIFFITHS, 2011). On the other hand, the tainting problems are due to the action of proteases, which generate bitter flavor, and lipases, which hydrolyze tributyrin and milk fat yielding free fatty acids, causing rancid, bitter, unclean, and soapy tastes (CROMIE, 1992; CHAMPAGNE et al., 1994; MANKAI et al., 2012).

'Age gelation' of UHT milk is an irreversible phenomenon characterized by a change in physical state, manifested by a rise in viscosity of more than 10 mPa.S (at 20°C) followed by the formation of a gel and loss of fluidity (DATTA; DEETH, 2001). This phenomenon is correlated to extensive protein breakdown caused by bacterial proteases on κ-casein yielding para-κ-casein in a manner similar to the action of rennet. A major difference is the lower specificity of bacterial proteinase compared to rennet which has a high specificity for the phe105-met106 bond of κ-casein (DATTA; DEETH, 2001). According to Sørhaug and Stepaniak (1997), a psychrotrophic population of 5.5 log CFU mL^{-1} in raw milk causes UHT milk gelation after 20 weeks of storage, while populations between 6.9 and 7.2 log will cause the same effect between 2 and 10 weeks.

2.3 Quorum Sensing and Metabolic Regulation

Bacteria communicate with each other via chemical signal molecules when specific cell densities are reached via a process termed quorum sensing (LIU; WANG; GRIFFITHS, 2007). As a consequence of this process, genes can either be activated or repressed and the activities of populations of single cells are synchronized, in a manner similar to that of multicellular organisms (SMITH; FRATAMICO; NOVAK, 2004; BAI; RAI, 2011).

Numerous studies have shown that cell density-dependent signaling systems in bacteria control a range of phenotypic traits, including biofilm development, bioluminescence, cell differentiation, competence for DNA uptake, pigment production, conjugal plasmid transfer, production of degradative extracellular enzymes, sporulation, toxin production and virulence gene expression (BAI; RAI, 2011).

The N-acyl-homoserine lactone (AHL) is a quorum sensing signal molecule which can be produced by a wide range of Gram-negative bacteria, including *P. fluorescens* (LIU; WANG; GRIFFITHS, 2007; OLIVEIRA; BRUGNERA; PICCOLI, 2010). The production of AHLs in both raw and pasteurized milk by psychrotrophic *Pseudomonas* spp. indicates that quorum sensing may play a role in the spoilage of milk and dairy products (PINTO et al., 2007).

Protease production by food spoilage *P. fluorescens* isolates is considered to be regulated by nutritional, temperature and aeration factors (MCKELLAR, 1989). Moreover, the production of extracellular proteases in *P. fluorescens* is also associated with the high cell density that is typically encountered towards the end of the exponential phase of growth (BAI; RAI, 2011).

Stimulation of protease production by milk associated strains of *P. fluorescens* in response to the addition of AHL has been demonstrated (LIU; WANG; GRIFFITHS, 2007). Moreover, it was shown that the degradation of AHLs, due to the addition of AHL-lactonase to *P. fluorescens* cultures, reduced protease production and that mutants defective in production of AHLs were also defective for the synthesis of protease (LIU; GRIFFITHS, 2003). Those authors concluded that the spoiling ability of psychrotrophic *P. fluorescens* was correlated with its ability to produce AHLs, which served to regulate the expression of extracellular proteases. In contrast, Pinto et al. (2010) did not detect AHLs signals in the supernatants of late-exponential and stationary phase broth cultures of *P. fluorescens* strain 07A, a strain isolated from milk. The authors subsequently added synthetic AHLs or bacterial extracts containing natural AHL's to O7A cultures and found no evidence for effects upon either growth or proteolytic activity, suggesting that quorum sensing (at least via AHL's) did not regulate protease production in strain 07A.

Although quorum sensing signaling molecules have been detected in cold stored milk and milk derivatives, the exact role played by them in the spoilage process is still not clear and further work on this topic is clearly warranted (BAI; RAI, 2011).

2.4 Biofilm Formation

Biofilms are surface-associated bacteria that are embedded in a matrix of self-produced polymeric substances (EPSs) consisting in nucleic acids, polysaccharides, lipids, and proteins, resulting of the successful attachment and subsequent growth of microorganisms on a surface (MARCHAND et al., 20012; TOYOFUKU et al., 2012).

They are a predominant form of growth for bacteria in the environment and in the clinic (MANN et al., 2012). The critical stages for the biofilm development are adherence, proliferation, and dispersion phases. Each of these stages includes reinforcement by, or modulation of, the extracellular matrix. *Pseudomonas* species utilize biofilm formation during plant colonization and environmental persistence, producing several biofilm matrix molecules (MANN et al., 2012).

In nature, biofilms can be composed of a single species or a consortium of numerous species. Some factors capable to influence them are: pH, nutrients availability, quorum sensing molecules, presence of organic and inorganic compounds and temperature (OLIVEIRA, 2010).

An important feature of milk-spoiling *Pseudomonas* recovered from biofilms is their ability to alter phenotype via the process of phase variation (MARCHAND et al., 2012). Through this process, reversible, high-frequency phenotypic switching is mediated by mutation, reorganization, or modification of the genome (VAN DEN BROEK et al., 2005), contributing to survival of the biofilm population during environmental stresses, such as temperature fluctuation, and frequent exposure to sanitizers during the cleaning of dairy processing equipment (MARCHAND et al., 2012).

2.5 Controlling Psychrotrophic Bacteria in Milk

The procedures of cooling and refrigerating of milk are no guarantees of quality. The first point of control is to ensure that raw milk is obtained under sanitary conditions designed to minimize contamination (BEALES, 2006). Clearly, higher initial counts of microorganisms in the raw milk increase the probability of changes in the final product (pasteurized or UHT milk and cheese) resulting from the action of psychrotrophic bacteria and their enzymes (MUIR, 1996).

The second point of control is in relation to adequate cleaning and disinfection of all equipment used for the collection, transport, and storage of refrigerated raw milk. This must be performed to prevent fouling with milk film, which can support growth of bacteria as multi-species biofilms that will represent a source of contamination for any subsequent batches of milk (MCPHEE; GRIFFITHS, 2011; PERIN et al., 2012).

Control of milk contamination may be achieved by the application of technologies including enclosed pipeline milk systems, better sanitary design of equipment, cleaner cows, and more effective "cleaning in place". These approaches coupled with the rapid cooling of raw milk using in-line plate coolers prior to storage in bulk tank has been shown to reduce the growth of contaminating bacteria (BARBANO; MA; SANTOS, 2006).

The control of microbial growth along a food processing line is also important and challenging. The availability of abundant nutrients and oxygen favor microbial growth, while the set-up of the processing line, which may include crevices, valves and gaskets, makes disinfection via regular sanitation procedures difficult and less efficient. Residues inherent to the processing of food accumulate in these locations provide a setting prone to colonization by bacteria, especially in the form of biofilms (CLETO et al., 2012).

Furthermore, it is noteworthy that if left untreated, water in contact with the milking equipment and containers can serve as a vehicle for contaminating microorganisms. The bacterial populations in such waters may appear to be insignificant in terms of CFU mL^{-1} in the milk, however, the multiplication of psychrotrophic bacteria in the waste water from

equipment can result in serious contamination through the establishment and development of undesirable microorganisms (COUSIN; BRAMLEY, 1981).

2.6 *Pseudomonas* in Milk

The genus *Pseudomonas* is the most heterogeneous and ecologically significant group of known bacteria. Owing to the fact that the nutritional requirements of *Pseudomonas* spp. are very simple, representatives of the genus have been detected in virtually all natural habitats e.g. soil, house dust, fresh water and clouds, but they have also been isolated from clinical instruments, aseptic solutions, cosmetics and medical products (FRANZETTI; SCARPELLINI, 2007).

As such, it is not surprising that members of the genus *Pseudomonas* have long been recognized as the predominant group of psychrotrophic bacteria recovered from spoiled refrigerated milk (MUIR, 1996; CHEN; DANIEL; COOLBEAR, 2003). Among the pseudomonads, *P. fluorescens* is often considered to be the principal spoilage agent of stored milk (MUNSCH-ALATOSSAVA; ALATOSSAVA, 2006; MCPHEE; GRIFFITHS, 2011).

These species are able to proliferate in milk, an environment where the concentration of free iron is low, due to the production of the diffusible fluorescent pigment pyoverdine, which acts as a siderophore, allowing the bacteria to effectively sequester iron from lactoferrin (MCPHEE; GRIFFITHS, 2011).

Pseudomonas isolated from milk showed generation times between 4-12 hours when inoculated in milk held at temperatures between 0 to 7°C (MUIR, 1996). The efficient cold adaption of the psychrotrophic pseudomonads is believed to be linked to the possession of elevated levels, between 59 to 72%, of unsaturated lipids in their cell membranes, which imparts the ability to efficiently maintain membrane functionality, specifically solute transport and secretion of extracellular enzymes, at refrigeration temperatures (JAY, 2005; FONSECA; MORENO; ROJO, 2011).

Studies on the behavior of *Pseudomonas* in cold stored milk have generated conflicting findings in relation to low temperature growth rates. According to De Jonghe et al. (2011), the difference between counts for *Pseudomonas* strains during prolonged storage under suboptimal (temp) and optimal (temp) storage conditions strains was two log cycles. Mcphee and Griffiths (2011) stated that *Pseudomonas* counts in the milk increase by only about one log cycle during the storage period. Unfortunately, direct comparisons between such studies are difficult to perform given methodological differences and the specific strains investigated. In this context, the importance of the choice of strain was highlighted in the work of by Jaspe et al. (1995), which demonstrated that *Pseudomonas* spp. isolated from milk that had been stored at 7°C for three days grew ten times faster at 7°C, had 1000 fold more proteolytic activity, and were 280 fold more lipolytic than *Pseudomonas* spp. isolated from freshly drawn milk.

Phenotypic analysis of microorganisms isolated from raw milk by Mcphee and Griffiths (2011) demonstrated that *P. fluorescens* biovar I (32.1% of isolates), *P. fragi* (29.6%), *P. lundensis* (19.8%), and *P. fluorescens* biovar III (17.3%) were the most commonly isolated species, and Marchand et al. (2009a) demonstrated that *P. lundensis* and *P. fragi* were the predominant milk spoilers in Belgian raw milk samples.

Similarly, He et al. (2009) found that pseudomonads predominated in cold stored, pasteurized milk at 10 and 5 days before expiration and on the expiration day, although they also detected significant numbers of *Streptococcus* spp. and *Buttiauxella* spp. in all samples.

Pseudomonads also predominated in the microbiota cultured from the crevices of cleaned devices sampled at a milk processing plant, demonstrating their potential role as post-collection contaminants (CLETO et al., 2012).

The ability of this group of microbes to resist cleaning is linked to the fact that many species are effective biofilm producers (SIMÕES; SIMÕES; VIEIRA, 2008; BAI; RAI, 2011). The complex and multilayered structures of the biofilm allow the bacterial communities to live in a sessile and protected environment. Yet, when population densities in biofilms become high, bacteria are released into the environment, providing a continuous source of planktonic bacteria capable of replication within the milk (BAI; RAI, 2011).

2.7 *Pseudomonas* spp.: Misidentification

A study of raw milk samples obtained along the cold chain of milk transportation (from farm, trucks, and silos) in Finland demonstrated that the majority (88%) of the isolated bacteria were psychrotrophic (MUNSCH-ALATOSSAVA; ALATOSSAVA, 2006). The authors employed two commercial phenotypic identification systems (API-20NE and BIOLOG GN2) and reported difficulties in obtaining confident identification of many of the isolates. This was particularly the case for fluorescent pigment producing pseudomonads, where the biochemical results were considered as doubtful. In view of this controversy, the authors recommended the use of genotypic identification systems in future studies.

A comparative evaluation of phenotypic and genotypic methods for the identification of 102 food associated psychrotrophic *Pseudomonas* spp. clearly demonstrated that the molecular methods provided superior results (FRANZETTI; SCARPELLINI, 2007). Thus, phenotypic data identified the bacteria as *P. fluorescens* or *P. putida*, with a single strain identified as *P. aeruginosa*. In contrast, sequencing of 16S rDNA in combination with restriction fragment length polymorphism (RFLP) analyses resulted in the identification of the bacteria as *P. jessenii*, *P. orientalis*, *P. migulae* and *P. chicorii* and also confirmed the phenotypic data for the *P. aeruginosa* isolate.

The study of Hantsis-Zacharov and Halpern (2007) evaluated 264 bacteria collected from raw milk during different seasons. They reported the presence of representatives of seven classes of bacteria (Gammaproteobacteria, Bacilli, Actinobacteria, Alphaproteobacteria, Betaproteobacteria, Flavobacteria and Sphingobacteria) with 20% of the isolates considered to be novel species. *Pseudomonas* and *Acinetobacter* were recorded as the predominant genera among the Gram-negatives with 33 and 29 isolates respectively. Sequencing of the gene encoding 16S rRNA identified the majority (15) of the isolates as *P. putida*, and demonstrated the presence of novel milk associated species *P. sinxantha*, *P. brenneri* and *P. veronii*.

Marchand et al. (2009a) employed a polyphasic approach, including molecular methods, for identification of the predominant producers of heat-resistant proteases in raw milk in Belgium. It was observed that *P. fragi* and *P. lundensis* represented 53% of the producers of heat-resistant proteases with *P. fluorescens* representing a minority of the isolates. In light of their results, the authors recommended the increased application of genotypic identification methods to ensure accuracy, and they also called for a revision of the taxonomic status of *P. fluorescens*. Furthermore, they considered it likely that misidentification of many proteolytic isolates as *P. fluorescens* in earlier studies using phenotypic characterization had led to an overestimation of the importance of this species as a milk spoiler.

A further example of the difficulties associated with the identification of milk spoiling *Pseudomonas* species was provided by the study of Corrêa et al. (2011). Using phenotypic methods, the authors identified the highly proteolytic *Pseudomonas* strain 1A4R as either *P. asplenii* or *P. jenssenii*. The subsequent application of 16S rDNA sequencing revealed a level of 99% nucleotide sequence homology with *P. koreensis*, which forms part of the so-called *P. jenssenii* group. In view of the divergent data, strain 1A4R was classified as a *Pseudomonas* sp. belonging to the *P. jenssenii* group.

2.8 Use of Molecular Tools to Elucidate Ecology of Psychrotrophic Bacteria

Indigenous raw milk bacterial communities, including potentially lipolytic and proteolytic psychrotrophs, are already present when the milk arrives at the dairy plant, making rapid and accurate identification of the raw milk microbiota a necessary prerequisite for the elaboration of methods to circumvent spoilage (VAN DER VOSSEN; HOFSTRA, 1996). Based on the observation that initial psychrotrophic counts of milk are frequently very low (MCPHEE; GRIFFITHS, 2011), more sensitive and efficient methods to evaluate the bacterial quality of raw milk are required in order to identify the causes of reduced shelf life and deterioration of technological properties of milk during storage.

Traditional microbiological approaches to the study of psychrotrophs in milk, based on phenotypic characterization, are time consuming, lacking discriminatory power and sensitivity and are often ineffective in establishing a causal relationship between contamination of the finished product and the environmental source (DOGAN; BOOR, 2003; RASOLOFO et al., 2010).

Furthermore, the inability to discriminate between closely related organisms can lead to misidentification and the slow turnaround of results makes phenotypic testing useful mainly for retrospective evaluation (ERCOLINI, 2004; RAATS et al., 2011). Molecular analyses of microbes offer some advantages over phenotypic methods, including speed and the ability to provide precise identification of microorganisms from genus to strain level, depending on the system used. In this context, discrimination between subspecies and strains is helpful for investigating routes and sources of contamination (ERCOLINI, 2004; RASOLOFO et al., 2010).

Molecular methods can be culture dependent (nucleic acids are recovered from cultured microbes), or culture independent, where total bacterial DNA/RNA is extracted directly from an environmental sample, providing information in relation to the components of the microbiota which are unable to grow under laboratory conditions (ERCOLINI, 2004; RAATS et al., 2011; ERCOLINI, 2013). The concept of viable but non-culturable (VBNC) bacteria refers to bacteria with metabolic activity and the ability to reproduce under suitable conditions, but which lack the capability to produce visible growth under standard conditions (OZCAKIR, 2007). Interest in the role of VBNC in food spoilage has increased due to the observation that some disinfection procedures such as pasteurization of milk and chlorination of water can cause bacteria to switch to the VBNC form (OZCAKIR, 2007).

The application of several culture dependent and independent methods applicable to milk and cheese was recently reviewed by Quigley et al. (2011). Among the culture independent methods, denaturing gradient gel electrophoresis (DGGE) which is based on the separation of complex mixtures of PCR amplicons of the same size but with different nucleotide sequences, has emerged as the most commonly used fingerprinting technique applied to the study of populations of psychrotrophics bacteria associated with milk and dairy

products (ERCOLINI, 2004; RASOLOFO et al., 2010; RAATS et al., 2011). This technology provides a convenient means to obtain a comprehensive overview of the microbial populations in milk during cold storage and has confirmed the predominance of *Pseudomonas* spp. in pasteurized milk samples during shelf life. Importantly, data from DGGE studies has also revealed a greater level of species diversity among the pseudomonads than had been indicated by culture (HE et al., 2009).

An alternative molecular fingerprinting method called random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) was applied to 66 bacterial isolates from cold stored raw milk, with subsequent nucleotide sequence analysis of the gene encoding 16S ribosomal RNA (ERCOLINI et al., 2009). Once again, this approach identified *Pseudomonas* spp. as the most common contaminant, but also identified *Hafnia alvei*, *Serratia marcersens*, *Citrobacter freundii*, *Staphylococcus* and *Lactococcus*.

3 CONCLUSION

The role of pseudomonads, principally *P. fluorescens*, as the predominant psychrotrophs associated with spoilage of cold stored milk and milk derived products has been established through a long history of culture based studies, often limited to the phenotypic characterization of the most abundant isolates following their isolation as pure cultures. The validity of this approach has been questioned by recent findings using nucleotide sequence based on identification methods, which have shown that the diversity of *Pseudomonas* species involved in milk spoilage is much wider than was previously thought.

The emergence of molecular methods provides a new way to obtain an accurate global view of microbial communities in milk. The application of these methods has revealed potential roles for genera other than *Pseudomonas* as important agents of milk spoilage at refrigeration temperatures. Moreover, it is likely that these mixed bacterial populations, often present in the form of biofilms, collaborate in the spoilage process via mechanisms including quorum sensing. The practical implications for these findings are not known yet. However, it is clear that continued study of the milk associated psychrotrophs is required and should be encouraged in order to enhance and improve existing control methods and help ensure the quality and safety of milk and milk derived foodstuffs.

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CAPÍTULO III

GENOTYPIC ANAYLSIS OF 16S rRNA GENE SEQUENCES COMPARED TO PHENOTYPIC IDENTIFICATION OF PSYCHROTROPHIC BACTERIA ISOLATED FROM MILK

ABSTRACT

Genotyping of 12 bacterial isolates recovered from raw milk in Brazil was performed based on sequencing and amplified ribosomal DNA restriction analysis (ARDRA) of an approximately 1500 base pair amplicon of the 16S rDNA gene. The bacterial had been identified phenotypically in a previous study as *Acinetobacter* spp. (n=4), *Aeromonas hydrophila* (n=3), *Pseudomonas fluorescens* (n=2) and *Pseudomonas putida* (3). ARDRA analysis using three restriction enzymes generated identical banding patterns for all three isolates received as *A. hydrophila*, for both of the *P. fluorescens* and for the three strains received as *P. putida*. Similarly, three of the four *Acinetobacter* isolates showed highly similar banding patterns for all three enzymes. However, the third *Acinetobacter* isolate (strain P162), produced a unique banding pattern with the enzyme *Sst*I. Sequencing of the 16S rDNA amplicons questioned the phenotypic identity of the two *P. fluorescens* isolates, and opened the possibility that they were in fact representatives of the species *P. rhodesiae*. In addition, sequencing confirmed the phenotypic identity of *A. hydrophila* for two of the aeromonads, with the third isolate (P65) showing a level of 99% sequence homology with sequences deposited as *A. punctata = A. caviae*. Species level identifications were determined for the four acinetobacters. Thus, strains P162, P164 and P165 were identified as *A. guillouiae*, while isolate P25 was identified as *A. johnsonii*. Interestingly, the three isolates received as *P. putida* showed 99% sequence homology with sequences deposited in the GenBank as *P. psychrophila,* species not previously reported in association with milk. Sequencing data was used to perform *in silico* digestion of the amplicons, with the program NEB Cutter Version 2.0 (New England Biolabs), employing the same three enzymes used in the ARDRA analysis and confirmed the banding patterns observed on gels. Finally, zymogram analysis of secreted proteases produced by the three aeromonads was performed using gelatin/polyacrylamide gels and demonstrated that the isolate P65 was different from the other two isolates. This observation provided support for the differential identification of this strain obtained via sequencing.

Keywords: *Aeromonas*, genotyping, sequencing

RESUMO

Foi realizada a genotipagem de 12 isolados bacterianos recuperados de leite cru no Brasil, com base no sequenciamento e na análise de restrição de DNA ribossomal amplificado (ARDRA) de cerca de 1500 pares de base do amplicon do gene 16S rDNA. As bactérias foram identificadas fenotipicamente em estudo anterior como *Acinetobacter* spp. (n= 4), *Aeromonas hydrophila* (n= 3), *Pseudomonas fluorescens* (n= 2) e *Pseudomonas putida* (n= 3). A técnica ARDRA com três enzimas de restrição gerou padrões de bandas idênticos tanto para os três isolados recebidos como *A. hydrophila* quanto para os dois recebidos como *P.*

fluorescens e para os três recebidos como *P. putida*. Similarmente, três dos quatro isolados de *Acinetobacter* mostraram padrões de bandas muito semelhantes para todas as três enzimas. No entanto, o terceiro isolado de *Acinetobacter* (linhagem P162) produziu padrão único de bandas com a enzima *Sst*I. O sequenciamento dos amplicons de 16S rDNA questionou a identidade fenotípica dos dois isolados de *P. fluorescens*, e abriu a possibilidade de que eles sejam, de fato, representantes da espécie *P. rhodesiae*. Além disso, o sequenciamento confirmou a identidade fenotípica de *A. hydrophila* para duas linhagens, com o terceiro isolado (P65) mostrando um nível de 99% de homologia com as sequências depositadas como *A. punctata = A. caviae*. Identificações em nível de espécie foram alcançadas para os quatro acinetobacters. Assim, as estirpes P162, P164 e P165 foram identificados como *A. guillouiae*, enquanto P25 isolado foi identificado como *A. johnsonii*. Curiosamente, os três isolados recebidos como *P. putida* mostraram 99% de homologia com sequências depositadas no GenBank como *P. psychrophila*, espécie não relatada anteriormente em associação com o leite. Foi realizada digestão *in silico* dos amplicons com o programa de NEB cutter Versão 2.0 (New England Biolabs) empregando as mesmas três enzimas usadas na técnica ARDRA, o que confirmou os padrões de bandas observados nos gel. Finalmente, a análise do zimograma de proteases produzidos pelas três *Aeromonas* foi realizada utilizando gel de gelatina/ poliacrilamida, que demonstrou que o isolado P65 era diferente dos outros dois isolados. Esta observação forneceu apoio para a identificação diferencial desta estirpe obtida através do sequenciamento.

Palavras-chave: *Aeromonas*, genotipagem, sequenciamento

1 INTRODUCTION

The microbiota of raw milk is directly influenced by the sanitary conditions of the animals at the time of collection and by the contamination control methods employed during storage and processing (MCPHEE; GRIFFITHS, 2011). Psychrotrophic bacteria are most often present as a minor component in freshly drawn milk when collected under good sanitary conditions (MARCHAND et al., 2009). However, the subsequent storage of the milk at cold temperatures provides a selective growth advantage to those microbes, which may result in spoilage of the milk and/or of milk derived products via the production of thermostable proteases and lipases (BEALES, 2006). Understanding the nature of, and the interactions between, the psychrotrophic bacteria in raw milk may serve to enhance and improve existing control methods and help ensure the quality and safety of milk and milk derived foodstuffs (RAATS et al., 2011).

Protease and lipase producing members the genus *Pseudomonas*, especially *P. fluorescens*, have long been considered as the predominant spoilage organisms associated with cold stored milk (MCPHEE; GRIFFITHS, 2011). Yet, data derived from the application of molecular microbiological methods has demonstrated that the actual composition of the psychrotrophic microbiota of raw milk is more complicated than was previously thought. Moreover, such data have served to stimulate research on other groups of milk associated bacteria which produce protease and lipases at cold storage temperature (HANTSIS-ZACHAROV; HALPERN, 2007).

The ability to arrive at meaningful conclusions in relation to the microbiology of milk spoilage can be hampered by deficiencies in our ability to accurately identify the bacteria isolated from that medium (MUNSCH-ALATOSSAVA and ALATOSSAVA (2006). Traditional phenotypic techniques for bacterial identification, based upon growth patterns on selective and differential media, biochemical reactions and microscopy, often lack discriminatory power and, as a consequence, misidentification, particularly at the species level, may occur (DOGAN; BOOR, 2003; KÄMPFER; GLAESER, 2012). Phenotypic methods make use of complex taxonomic keys which are based upon results from type cultures and which will often fail to identify biochemically aberrant strains of the same species. In addition and from a practical viewpoint, results may be difficult to reproduce between laboratories and the slow turnaround time for elaboration of complete phenotypic characterization makes such data useful mainly for retrospective evaluation (VAN DER VOSSEN; HOFSTRA, 1996). For those reasons, genotypic identification methods have been increasingly used as an alternative or complement to established phenotypic procedures (MARCHAND et al., 2009).

In the 1980s, the notion of a new standard for identifying bacteria began to be developed based on comparing a stable region of the genetic code. The nucleotide sequence which is now most commonly used for taxonomic purposes for bacteria is the 16S rDNA gene, which encodes the small subunit of prokaryotic ribosomal RNA and, for this, also referred as 16S rRNA (CLARRIDGE, 2004; KÄMPFER; GLAESER, 2012). The invention of the polymerase chain reaction (PCR) and automated DNA sequencing, has led to the accumulation of a vast amount of sequence data on the genes encoding 16S rRNA in prokaryotes, which is stored in publically accessible databases including the GenBank, maintained by the National Centre for Biotechnological Information (NCBI), in the United

States of America (BENSON et al., 2012). Comparative analyses of these sequences using search algorithms such as the Basic Local Alignment Search Tool "BLAST" (ALTSCHUL et al., 1990)**,** has shown that the rRNA gene sequences are highly conserved within living organisms of the same genus and species, but that they differ between organisms of other genera and species. Therefore, using 16S rRNA sequence analysis, numerous bacterial genera and species have been reclassified and renamed, classification of uncultivable bacteria has been determined, and the discovery and classification of novel bacterial species has been facilitated (WOO et al., 2008). It should be noted that analysis of 16S rRNA sequences represents a powerful, but not infallible, tool that has been used to trace phylogenetic relationships between bacteria, and to identify bacteria from various sources, such as environmental or clinical specimens (MIGNARD; FLANDROIS, 2006; JANDA; ABBOTT, 2007).

Despite continuing reductions in the cost of sequencing methods, the routine application of sequencing as a method for bacterial identification is still not financially viable for many laboratories. In light of this restraint, alternative and less expensive methods for analysis of 16S rRNA are commonly used as a means of performing preliminary molecular analysis of isolates and for screening culture collections to select representative isolates for sequencing. One of the most frequently employed techniques is amplified ribosomal DNA restriction analysis (ARDRA), which relies upon restriction endonuclease based digestion of a PCR amplified region of the 16S rRNA gene to generate a series of differently sized fragments (RAMESHKUMAR et al., 2012). The digested DNA is separated electrophoretically in gels (agarose or polyacrylamide) (BENDALL et al., 1995; GIL-LAMAIGNERE et al., 2003) and the image of the gel is recorded digitally for posterior analysis of banding patterns. Variations in banding patterns among strains can occur as a result of mutations in restriction site sequences, acquisition or deletion of recognition sites, or insertions and deletions in the sequences between recognition sites (GIL-LAMAIGNERE et al., 2003).

In the current study, a total of 12 Gram-negative psychrotrophic bacteria previously isolated from raw milk in Brazil and identified using phenotypic methods (ARCURI et al., 2008) were classified using a combination of ARDRA and sequencing of near full-length 16S rDNA amplicons.

2 MATERIAL AND METHODS

2.1 Bacteria and Growth Conditions

A total of 12 bacterial cultures comprising four strains of *Acinetobacter* spp. (P25, P162, P164 and P165), three *Aeromonas hydrophila* (P07, P10 and P65), two *Pseudomonas fluorescens* (P88 and P250) and three *Pseudomonas putida* (P270, P272 and P273), all isolated from raw milk, were provided by the microbial network of the National Platform of Genetic Resources (CA5) located at the Brazilian Agricultural Research Corporation (EMBRAPA) - Dairy Cattle division (Juiz de Fora, Minas Gerais, Brazil). The cultures had previously been identified phenotypically using the API 20E and API 20NE systems (bioMérieux, Marcy l'Etoile, France) as reported by Arcuri et al. (2008). Stock cultures were maintained at -25°C in tryptone soya broth (TSB) (Oxoid, Basingstoke, UK) supplemented with 20% (vol/vol) glycerol and in nutrient agar slants held at 4°C. Working cultures were produced by inoculation of tryptone soya broth with incubation for 24h at 30°C, followed by subculture onto plates of nutrient agar, with incubation for 24h at 30°C, to obtain single colonies.

2.2 Polymerase Chain Reaction Amplification of 16S rDNA

DNA for use as template in the PCR was obtained by thermal lysis. Specifically, well isolated, single colonies of each isolate were resuspended in 350 µL of molecular biology grade water (Sigma-Aldrich, St. Louis, MO, USA), in individual 1.5 mL screw capped micro centrifuge tubes (Axygen, Union City, CA, USA) and were placed in a boiling water bath for 5 min. The lysates were subsequently centrifuged (16.000 x *g*) to sediment cell debris and duplicate 100 µL volumes of supernatant were transferred to new tubes, with storage at -20°C.

PCR based amplification of an approximately 1530 bp fragment of the 16S rDNA gene was performed using the universal primers AMP1 (5' GAG AGT TTG ATY CTG GCT CAG 3') and AMP2 (5' AAG GAG GTG ATC CAR CCG CA) on a Mastercycler Gradient Thermocycler (Eppendorf, Hamburg, Germany). PCR reaction mixes comprised, 1 µL of DNA, 2.5 µL of 10X reaction buffer (Invitrogen, Paisley, UK), 3mM $MgSO_4$ (Invitrogen), 200 µM of each dNTP (Promega, Madison, WI, USA), 3.75 pmol of each primer and 0.5 unit of Platinum® Taq Polymerase High Fidelity (Invitrogen); molecular biology grade water was used to adjust the final reaction volume to 25 µL. Negative controls containing 1 µL of molecular biology grade water, in the place of template, were included in each assay. The cycling conditions were: an initial denaturation at 95 °C for 150s, followed by 33 cycles of 95 °C/15s, 60°C/45s and 68°C/120s; with a final extension at 68°C/5min.

2.3 Purification and Quantification of Amplicons

The amplicons of 16S rDNAs were purified using the GFX™ illustra PCR and Gel Band Purification Kit (GE Healthcare Life Sciences, Uppsala, Sweden), following the recommendations of the manufacturer and subsequently quantified spectrophotometrically using a NanoDrop® 2000 apparatus (Thermo Fisher Scientific, Waltham, MA, USA). In order to confirm the fidelity of the amplification reactions, i.e., the presence of a single amplicon of the predicted size, electrophoresis was conducted using 1% agarose gels prepared in 1X Tris

Acetate EDTA (TAE) buffer at a constant voltage of 5V/cm for 60 min. The molecular size marker employed was a 100 base pair ladder (New England BioLabs, Ipswich, MA, USA). Gels were stained with ethidium bromide for 10 min, destained in water for 30 min, observed and digitally photographed under ultra-violet illumination in a Gel Doc XR photo-documenter (Bio-Rad, Hercules, CA, USA) and the image was analyzed using Quantity-One software (Bio-Rad). In addition, samples used in sequencing reactions were quantified on agarose (1%) gels by comparison with a Low Mass DNA Ladder (Invitrogen).

2.4 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Restriction digestion of each amplified 16S rDNA was performed using three restriction endonucleases: *Hae*III, *Hinf*I e *Sst*I (all from Invitrogen). Digestion mixtures contained approximately 500 ng of PCR amplicon (calculated based on the concentration values derived spectrophotometrically by NanoDrop®), 1.2 µL of the appropriate reaction buffer, 1 µL (10 units/µl) of restriction endonuclease and molecular biology grade water to achieve a final volume of 12 µL. Reaction tubes were incubated at 37°C for 2 h, after which 3 µl of 6X gel loading buffer (Invitrogen) was added to each tube, followed by electrophoresis in agarose gels (1.5%) at a constant voltage of 5V/cm for 135min. All gels contained 100 bp DNA ladder (N32315 New England Bio Labs). Banding patterns were compared between isolates of the same species and in comparison to the 100 bp ladder to estimate the size of restriction fragments.

2.5 Sequence Analysis

Purified PCR amplicons were sequenced in both directions using the amplification primers and six additional internal sequencing primes (Table 1), to provide unambiguous sequence data by use of the DYEnamic ET Dye Terminator Kit (MegaBACE) (GE Healthcare); reaction products were analyzed on a MegaBace™ 1000 sequencer (GE Healthcare). Sequence alignments were performed using the programme Sequencher Version 5.0 (Genecodes Corporation, Ann Harbor, MI, USA). Aligned sequences were entered into the BLAST search algorithm and the NCBI nucleotide database to determine gene identity. *In silico* restriction digest of the sequences was performed using the on-line program NEBcutter V2.0 developed by Vincze; Posfai and Roberts (2003) and available at tools.neb.com/NEBcutter2/. The results of these analyses were compared with the ARDRA data.

Table 1. Primers employed in the sequencing reactions (continued on the next page…)

Primer name	Oligonucleotide conditions	Direction
AMP1	GAG AGT TTG ATY CTG GCT CAG	Forward
AMP2	AAG GAG GTG ATC CAR CCG CA	Reverse
786F	CGA AAG CGT GGG GAG CAA ACA GG	Forward
RU6	ATG GCT GTC GTC AGC TCG T	Forward
1110R	TGC GCT CGT TGC GGG ACT TAA CC	Reverse
362F	CTC CTA CGG GAG GCA GTG GGG	Forward
U3	GWA TTA CCG CGG CKG CTG	Reverse

Table 1. Continuation.

Primer name	Oligonucleotide conditions	Direction
U2	TGC TGC CTC CCG TAG GAG	Reverse

2.6 Zymogram Analysis of Extracellular Proteases of *Aeromonas* spp.

Starter cultures of the three *Aeromonas* isolates were prepared by inoculating 10 mL of TSB with an individual colony followed by incubation overnight at 30°C. Aliquots (100 µL) of the starter cultures were used to inoculate fresh 10 mL volumes of TSB with incubation at 30°C for 24 h. Cell free extracts were prepared by centrifugation (8000 x *g* for 10 min), followed by filtration (0.22 µM) of 8 mL of culture supernatant. The protein concentration was determined spectrophotometrically (NanoDrop® 2000, Thermo Scientific).

Protease activities were assayed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 0.1% co-polymerized gelatin as substrate. Gels were loaded with 50 µg of protein per slot. After electrophoresis, at a constant current of 120 V for 120 min, SDS was removed by incubation with 10 volumes of 2.5% Triton X-100 for 1 h at room temperature under constant agitation. In order to promote proteolysis, the gels were incubated for 24 h at 30°C in 20 mM glycine-NaOH buffer (pH 9.0). The gels were stained for 2 h with 0.2% Coomassie® Brilliant Blue R-250 (Sigma-Aldrich) in methanol:acetic acid:water (50:10:40) and destained overnight in a solution containing methanol:acetic acid:water (5:10:85) to intensify the digestion halos. The gels were dried, scanned and digitally processed using Quantity-One software (Bio-Rad).

3 RESULTS

3.1 PCR

As shown in Figure 1, DNA extracted from each of the bacterial species generated a single amplicon of approximately 1500 base pairs.
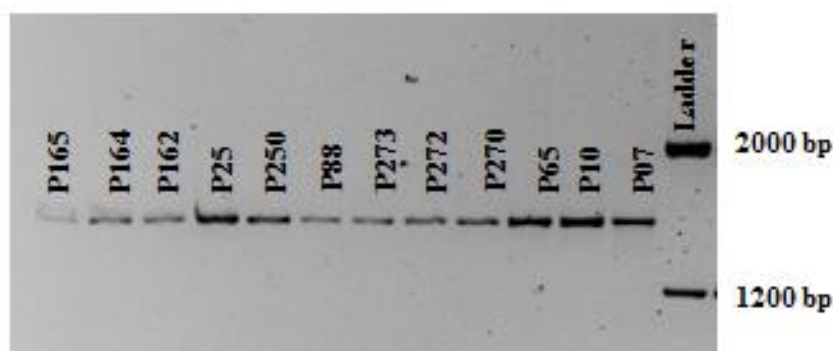
Figure 1. Agarose gel (1%) showing amplification of an approximately 1500 bp fragment of 16S rDNA for each one of the test bacteria, using the primers AMP1 and AMP2. Ladder= Low DNA Mass Ladder (Invitrogen).

3.2 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

The banding patterns generated for each species following digestion with the three test enzymes (*Hae*III, *Hinf* I e *Sst*I) are provided in Figure 2 and Figure 3. It should be noted that for each isolate a control digestion comprising DNA, buffer and water (but without restriction enzyme) was included to confirm the presence of artifactual bands. It can be observed that the staining intensity of the undigested DNA varied between samples, showing that the quantification performed using the NanoDrop® apparatus had overestimated the quantity of DNA in some samples, e.g. the first three *Acinetobacter* strains (resulting in weak banding patterns), and conversely had underestimated the quantity of DNA in other samples e.g. the three Aeromonads (resulting in overtly intense bands). The reasons for this are unclear, but the production of unreliable quantification is well recognized for the NanoDrop® apparatus. Despite this shortcoming it was still possible to perform meaningful comparison between the isolates.

In the case of the Acinetobacters, all four strains produced identical banding patterns when digested with *Hae*III and *Hinf*I. The pattern generated by digestion with *Hae*III was complex and comprised a single band of approximately 350 base pairs (bp), a doublet of bands of approximately 260bp, a single band of approximately 180bp and a group of 3 or 4 bands which clustered together just below the 100bp (Figure 2). The *Hinf*I banding pattern was simpler to interpret and comprised a single band of approximately 950bp, a doublet of bands of approximately 220bp and a single band of approximately 180bp (Figure 2). The enzyme *Sst*1 did not cut the amplicons generated by strains P25, P164 and P165. However, a single restriction site for this enzyme was observed in strain P162, resulting in the presence of two bands of approximately 1100bp and 420bp (Figure 2). The combined results indicated that the four strains were closely related. The interpretation of the banding patterns for some

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digests was complicated by the presence of partially digested DNA. This feature was most prominent for the digests produced with *Hinf*I, and was particularly notable in the case of strain P165 (Figure 2). Non-specific artifacts were also observed with *Sst*I, but the same bands were observed in the control (enzyme free digests). The pattern generated for strain P165 (multiple high molecular weight bands) was most likely explained by leakage of sample from the neighboring wells (Figure 2).

The banding patterns recorded for the two putative *P. fluorescens* strains were identical for all three digests. Digestion with *Hae*III generated a single band of approximately 440 bp, a doublet of bands of approximately 250bp, an additional doublet of approximately 170 bp and a complex of faint bands below the level 100 bp of the marker. The *Hinf*I banding pattern was made up of a single band of approximately 1000bp, a single band of approximately 200bp and a doublet of approximately 120bp. Both of the *P. fluorescens* amplicons possessed a single site for the enzyme *Sst*I, resulting in the production of two bands of approximately 850 and 700 bp (=1550 bp). In addition, both *Sst*I digests contained a band of approximately 500 bp (Figure 2), but those bands were clearly artifacts.

Identical banding patterns were observed for all three putative strains of *P. putida* with each of the three enzymes (Figure 3). Data which, once again, suggested close relationship between the isolates. Interestingly, the *P. putida* digests were notable for the lack of non-specific bands or partial digestion which was recorded in the case of the other bacteria (Figure 2 and Figure 3). The *Hae*III banding pattern consisted of single bands of approximately 670bp and 230bp in combination with a triplet of bands of approximately 170bp (Figure 3). Digestion with *Hinf*I generated single bands of approximately 1000bp and 210bp in conjunction with a doublet of fragments of approximately 120bp. The enzyme *Sst*I did not cut any of the three *P. putida* amplicons.

Based on ARDRA data, a high degree of relatedness was also suggested for the three putative *A. hydrophila* isolates. In common with results recorded with the *P. putida* amplicons, the enzyme *Sst*I did not cut any of the *A. hydrophila* derived sequences. On the other hand, digestion with *Hae*III and *Hinf*I produced identical banding patterns, which were somewhat difficult to analyze owing to the excessive quantity of DNA which was employed in the digestion mixtures. Digestion with *Hae*III revealed a single band of approximately 320bp, a doublet of approximately 220bp, and a group of three or possibly four bands of approximately 150 to 160bp (Figure 3). Analysis of *Hinf*I digest showed the presence of a triplet of bands of 320 to 350bp in size, and single bands of approximately 245bp and 140bp.
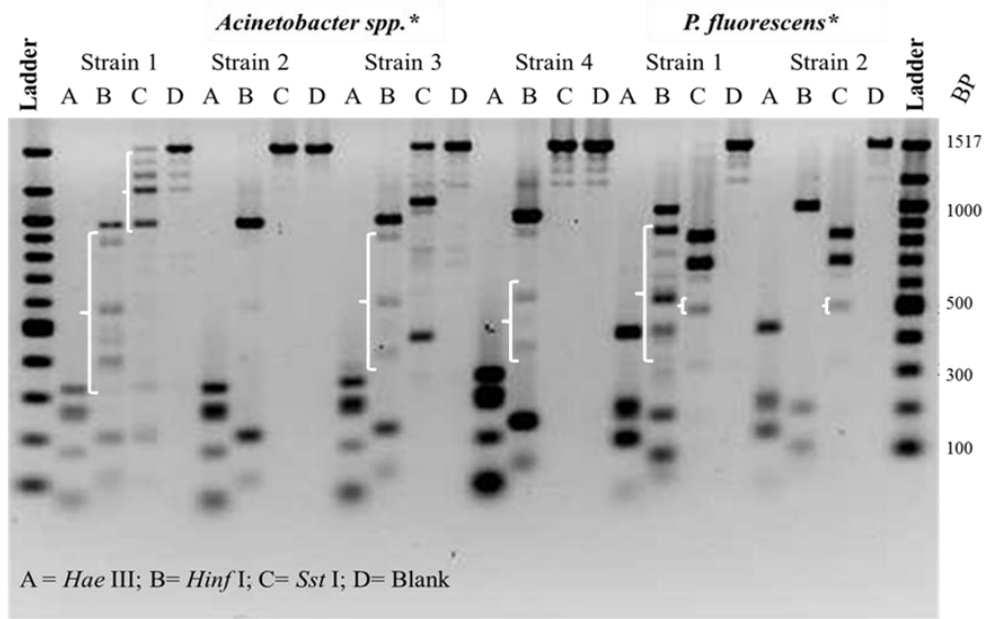
Figure 2. ARDRA banding patterns generated for bacterial isolates identified phenotypically* as *Acinetobacter* spp. or as *Pseudomonas fluorescens*. The restriction enzymes used were (A) *Hae*III (B), *Hinf*I and (C) *Sst*I, and (D) represents control DNA which was not treated with any enzyme. The strains examined were *Acinetobacter* spp. (1= P165, 2= P164, 3= P62, 4= P25) and *P. fluorescens* (1= P250, 2= P88). The sizes of digest fragments were estimated in comparison to a molecular weight Ladder (100 bp DNA ladder; product number N3231S New England Bio Labs), included in the first and last lanes of the gel, with representative sizes noted on the right hand extremity of the gel image. Areas of partial digestion and or artifactual bands were highlighted with "{".
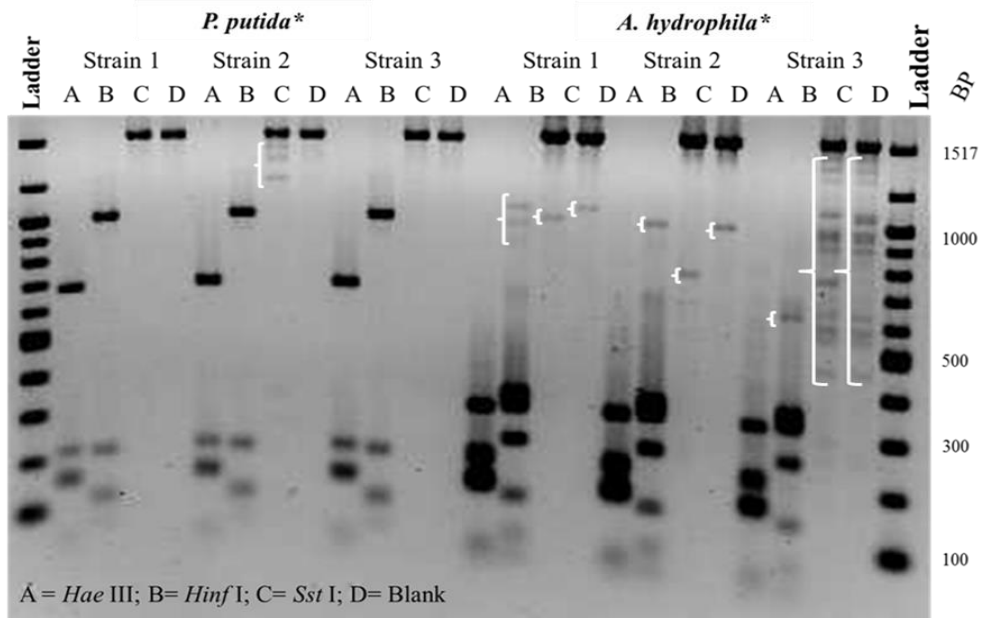
Figure 3. ARDRA banding patterns generated for bacterial isolates identified phenotypically* as *Pseudomonas putida* or as *Aeromonas hydrophila*. The restriction enzymes used were (A)

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*Hae*III (B), *Hinf*I and (C) *Sst*I, and (D) represents control DNA which was not treated with any enzyme. The strains examined were *P. putida* (1= P273, 2= P272, 3= P270) and *A. hydrophila* (1= P65, 2= P10, 3= P07). The sizes of digest fragments were estimated in comparison with molecular weight Ladder (100 bp DNA ladder N3231S New England Bio Labs), included in the first and last lanes of the gel, with representative sizes noted on the right hand extremity of the gel image. Areas of partial digestion and or artifactual bands were highlighted with "{".

3.3 Sequence Analyses of 16S rDNA Amplicons

High quality sequencing data (as indicated by clearly defined single peaks for each nucleotide in the chromatograms) were obtained for each of the twelve 16S rRNA amplicons. Sequence alignment resulted in contigs ranging in size from 1490 to 1445 bp. The results of submission of the sequences to the BLAST search algorithm and the NCBI nucleotide database to determine gene identity are presented in Table 2. Therein, the top three hits in the GenBank are shown for each of the twelve tested bacteria, along with the percentage nucleotide homology.

In the case of the Acinetobacters, comparative sequence analysis revealed that two strains (P164 and P165) showed 99% sequence homology with *A. guillouiae* ATCC 11171 and that strain P162 showed 98% sequence homology with the same type strain (Table 2). Alignment of the P164 and P165 sequences using the program Sequencher 5.0 showed them to be essentially identical having only two nucleotide difference over a total of 1489 nucleotides sequenced. Alignment of the sequences derived from P164 and P165 with that of P162 demonstrated a total of 34 nucleotide differences over 1492 base pairs sequenced. These differences were concentrated in the hypervariable regions V1 and V3 (CHAKRAVORTY et al., 2007). The fourth *Acinetobacter* isolate (P25) showed 99% sequence homology to *A. johnsonii* ATCC 17909. Alignment of this sequence with those of P164 and P165 revealed a total of 44 nucleotide differences over 1493 base pairs of sequence. A greater degree of difference i.e. 54 nucleotides in 1489 was observed when the P25 sequence was aligned with the P162 sequence.

Both of the isolates phenotypically identified as *P. fluorescens* were shown to have 99-98% sequence homology with sequences deposited in the GenBank as *P. rhodesiae*, (Accession number NR024911.1). In the case of isolate P88, the top three matches highlighted in the BLAST search were with sequences deposited as *P. rhodesiae* (Table 2). On the other hand, isolate P250 also showed 98% homology with sequences deposited as *P. fluorescens* (GenBank Accession number; GU198125.1) and as *Pseudomonas sp.* HC1-30 (Accession number JF312942.1). A comparative alignment between the two isolates showed a total of 21 nucleotide differences over 1455 base pairs of sequence. The majority of the differences were single nucleotide polymorphisms, but a cluster of 13 base pair differences was observed in the V6 hypervariable region.

The majority (7 of 10 hits) identified in BLAST searches performed using the sequences derived from the three *P. putida* isolates (P270, P272 and P273) were classed as uncultured bacterial clones, which, despite showing 99% nucleotide sequence homology, did not help to resolve the identity of the test isolates. The remaining three hits were all deposited as *P. psychrophila* (Table 2), and each showed 99% sequence homology with the three isolates phenotypically identified as *P. putida*. An alignment of the three novel sequences demonstrated that they were highly similar and showed only eight nucleotide differences over

a total of 1494 sequenced base pairs. The nucleotide differences were randomly distributed throughout the sequence and were all single nucleotide polymorphisms.

Comparative analysis of the sequences derived from the three aeromonads confirmed the phenotypic identity of isolates P07 and P10 as *A. hydrophila*. Alignment of these sequences using Sequencher 5.0 showed them to be near identical (only 3 nucleotide differences over 1494 sequenced base pairs). In contrast, isolate P65 was identified as showing 99% homology with sequences deposited as *A. punctata = A. caviae* (JANDA; ABBOTT, 2010). Alignment of this sequence with those of P07 and P10 revealed a total of 18 nucleotide differences in a total of 1494 sequenced nucleotides, with the majority (11 of 18) clustered in the V3 hypervariable region.

The twelve sequences deduced in this study have been deposited in the NCBI nucleotide database with the accession numbers provided in Table 3.

Table 2. Relationship of 16S rDNA sequences amplified from the phenotypically identified bacteria to other sequences in GenBank (continued on the next page…)

Phenotypic ID	Strain	Highly similar sequences	Accession n.	% Homology
Acinetobacter spp.	P165	*A. guillouiae* ATCC 11171	HE651924.1	99
		A. guillouiae strain FFL26	JN092611.1	99
		Acinetobacter sp.	EU341175.1	99
	P164	*A. guillouiae* ATCC 11171	HE651924.1	99
		A. guillouiae strain FFL26	JN092611.1	99
		Acinetobacter sp.	EU341175.1	99
	P162	*A. guillouiae* ATCC 11171	HE651924.1	98
		A. guillouiae	JN092611.1	98
		Acinetobacter sp.	HQ530522.1	98
	P25	*A. johnsonii* ATCC 17909	HE651920	99
		Acinetobacter sp.	JN082564.1	99
		Acinetobacter sp.	JN082538.1	99
P. fluorescens	P88	*P. rhodesiae* strain NO5	FJ462694.1	99
		P. rhodesiae CIP 104664	NR024911.1	99
		P. rhodesiae strain: 65b	AB698741.1	98
	P250	*P. rhodesiae* CIP 104664	NR024911.1	98
		P. fluorescens	GU198125.1	98
		Pseudomonas sp. HC1-30	JF312942.1	98
P. putida	P273	*P. psychrophila* SAs-12	JQ968689.1	99
		P. psychrophila HA-4	JQ968688.1	99
		P. psychrophila Den-03	JQ782901.1	99
	P272	*P. psychrophila* SAs-12	JQ968689.1	99
		P. psychrophila HA-4	JQ968688.1	99
		P. psychrophila Den-03	JQ782901.1	99

Table 2. Continuation.

Phenotypic ID	Strain	Highly similar sequences	Accession n.	% Homology
		P. psychrophila SAs-12	JQ968689.1	99
	P270	*P. psychrophila* HA-4	JQ968688.1	99
		P. psychrophila Den-03	JQ782901.1	99
		A. punctata RK 65541	AY987761.1	99
	P65	*A. punctata* AE-34	AY987727.1	99
		Aeromonas sp. AN-24	AY987738.1	99
A. hydrophila		*A. hydrophila*	NR043638.1	99
	P10	*A. hydrophila*	NR074841.1	99
		Aeromonas sp.	GU566305.1	99
		A. hydrophila	GQ184148.1	99
	P07	*A. hydrophila*	NR043638.1	99
		A. hydrophila	NR074841.1	99

Table 3. GenBank accession numbers of the 16S rDNA amplicons of the tested isolates.

Strain	Identified by sequencing as	GenBank Accession number
P165	*A. guillouiae*	KC904085
P164	*A. guillouiae*	KC904086
P162	*A. guillouiae*	KC904087
P25	*A. johnsonii*	KC904088
P250	*P. rhodesiae*	KC904089
P88	*P. rhodesiae*	KC904090
P273	*P. psychrophila*	KC904091
P272	*P. psychrophila*	KC904092
P270	*P. psychrophila*	KC904093
P65	*A. punctata*	KC904094
P10	*A. hydrophila*	KC904095
P07	*A. hydrophila*	KC904096

The results of the *in silico* digests performed with the program NEBcutter 2.0 are provided in Table 4.

Table 4. Fragments generated by *in silico* digestion of 16S rDNA amplicon sequences using the program NEBcutter 2.0. a) *Acinetobacter* spp.* (P165, P164, P162 and P25) no compared with a reference strain; b) *P. fluorescens** (P250 and P88) compared with a reference strain; c) *P. putida** (P273, P272, P270) compared with a reference strain; d) *A. hydrophila** (P10, P07 and P65) compared with a reference strain (Continued on the next page…)

a)	P165		P164		P162			P25	
Fragments	*Hae*III	*Hinf*I	*Hae*III	*Hinf*I	*Hae*III	*Hinf*I	*Sst*I	*Hae*III	*Hinf*I
1	326	974	326	975	328	976	1073	326	977
2	278	211	278	211	278	209	423	279	209
3	236	179	236	182	236	182		235	172
4	180	96	180	99	180	101		180	97
5	96	15	96	15	96	15		96	15
6	91	13	92	13	91	13		93	13
7	89		89		89			89	
8	85		85		85			85	
9	66		66		66			66	
10	41		47		47			34	

b)	*P. fluorescens* LMG5167			P250			P88		
Fragments	*Hae*III	*Hinf*I	*Sst*I	*Hae*III	*Hinf*I	*Sst*I	*Hae*III	*Hinf*I	*Sst*I
1	845	1002	823	438	988	818	440	1002	816
2	220	209	679	267	209	630	242	209	674
3	171	120		242	119		220	120	
4	139	103		171	107		171	96	
5	59	51		162	17		161	46	
6	34	17		98	8		134	17	
7	25			34			59		
8	9			27			34		
9				9			20		
10							9		

Table 4. Continuation.

c)	*P. putida* KT2440		P273		P272		P270	
Fragments	*Hae*III	*Hinf*I	*Hae*III	*Hinf*I	*Hae*III	*Hinf*I	*Hae*III	*Hinf*I
1	682	503	670	1005	682	1001	682	1002
2	220	499	220	209	219	209	220	209
3	171	209	171	120	171	120	171	120
4	161	120	161	98	161	98	161	95
5	151	75	139	48	135	44	136	48
6	59	63	59	17	59	17	59	17
7	34	32	35		34		34	
8	31	17	19		19		19	
9	9		14		9		9	
10			9					
d)	*A. hydrophila* ATCC 7966		P10		P07		P65	
Fragments	*Hae*III	*Hinf*I	*Hae*III	*Hinf*I	*Hae*III	*Hinf*I	*Hae*III	*Hinf*I
1	682	503	317	348	317	348	317	349
2	220	499	220	317	220	317	220	317
3	171	209	204	313	204	312	204	312
4	161	120	171	245	171	245	171	245
5	151	75	169	137	169	137	169	138
6	59	63	160	46	160	42	161	42
7	34	32	134	32	130	32	131	32
8	31	17	59	31	59	31	59	31
9	9		34	15	34	15	34	15
10			20	13	19	13	19	13
11			9		9		9	

*According to the phenotypic identity. Reference strains: *P. fluorescens* LMG5167 (GenBank accession number GU198104.1); *P. putida* KT2440 (GenBank accession number; NR-043638.1) and *A. hydrophila* ATCC 7966 (GenBank accession number; NC-002947.3).

3.4 Zymogram Analysis of Extracellular Proteases of *Aeromonas* spp.

A qualitative comparison of the proteases present in the extracellular growth medium of isolate P65, P07 and P10 is presented in Figure 4 . It can be seen that isolate P65 presented a different banding pattern (where bands represent areas of proteolytic activity towards gelatin) than that seen for isolates P07 and P10. Specifically, isolate P65 generated three clear areas of degradation of gelatin (A, B and C). The other two isolates showed identical banding patterns comprising four areas of gelatin breakdown (D, E, F and G).

Lane 1= P65, Lane 2= P07 and Lane 3= P10

Areas of proteolytic activity (A to G) are highlighted.
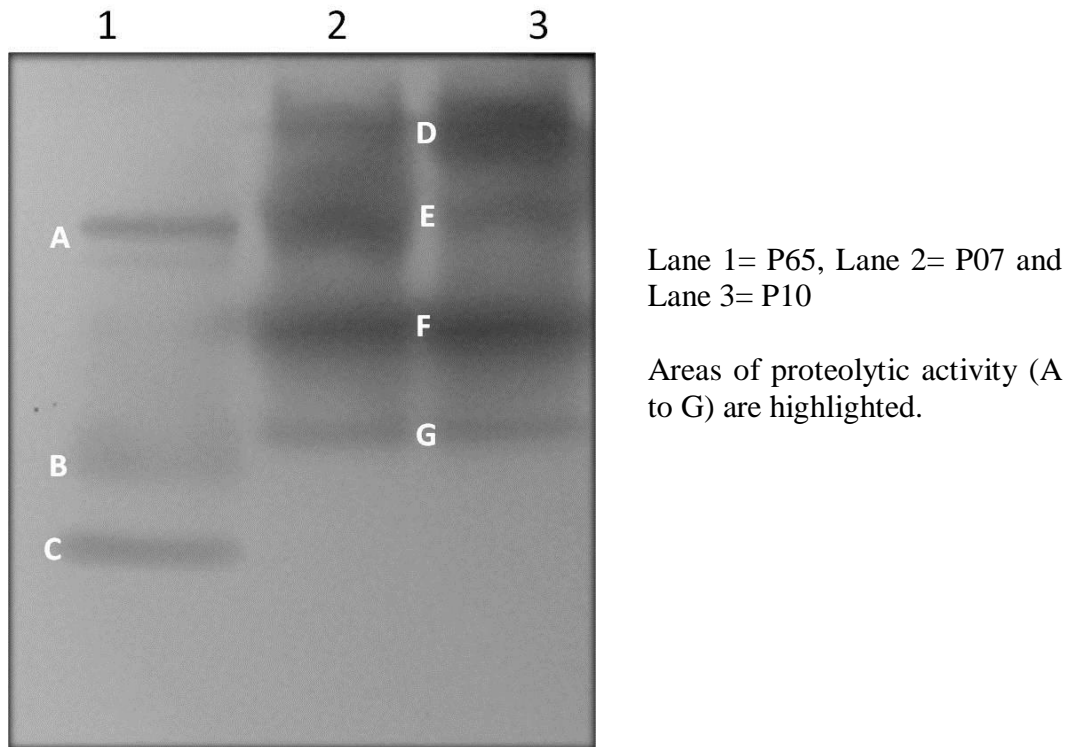
Figure 4. Proteolytic activity of electrophoretically separated extracellular products of the three test strains of *Aeromonas*.

4 DISCUSSION

The experiments conducted in this study sought to employ molecular methods to either confirm or repudiate data in relation to the phenotypic classification of 12 psychrotrophic bacteria previously isolated from Brazilian milk (ARCURI et al., 2008). Clearly, the correct designation of organisms is important. For example, when we refer to a complex of organisms by a single name and these organisms have different biochemical potential, the microbiological process associated with those bacteria (e.g. food spoilage) may be obscured. Hence, the planned confirmation of identity represented the essential first step in a study investigating the ability of the bacteria to produce caseinmacropeptide (CMP) and pseudo CMP at cold storage temperatures. Until now, this feature has been studied exclusively using psychrotrophic isolates of *P. fluorescens* (RECIO et al., 1996; RECIO et al., 2000; MOTTA; HOFF, 2009; OLIVEIRA et al., 2009). In order to extend the range of possible CMP producers we included a total of ten isolates phenotypically identified as *Acinetobacter* spp., *Aeromonas hydrophila* and *Pseudomonas putida*, in addition to two strains characterized as *P. fluorescens* (ARCURI et al., 2008).

It would be fair to state that phenotypic data have formed and, in some laboratories, continue to form the traditional and only basis for the characterization of microorganisms. Phenotypic traits comprise the observable characteristics that result from the expression of genes of an organism (KÄMPFER; GLAESER, 2012). However, environmental conditions (e.g. pH, oxygen tension, temperature) can modulate which genes are expressed and also the degree to which they are expressed. Thus despite appearing to be a straightforward process, involving the recognition of simple features, the mechanisms controlling the expression of the phenotypic traits are in fact very complex. In addition, the requirement for standardization in terms of the methods and material used can make the comparison of data generated from different laboratories a difficult task. As a result of these dependencies, many methods targeting the complex phenotypic characteristics of prokaryotes suffer from differing degrees of reproducibility and it is essential that rigorous standardization for the analysis of phenotypic data is applied (TINDALL et al., 2010; KÄMPFER; GLAESER, 2012).

In contrast to the phenotypic traits, genotypic traits of an organism are those stored in the genome i.e. the entirety of an organism's hereditary information. The development of DNA-based analyses for the characterization of microorganisms has progressed steadily during the last 50 years (SENTAUSA; FOURNIER, 2013). Initially, genotypic analyses were limited to comparisons of the guanine and cytosine (G+C) ratio within the total genomic DNA (KÄMPFER; GLAESER, 2012). This was followed by the development in the 1970's of techniques based upon the DNA–DNA hybridization of genomic DNA (ROSSELLÓ-MORA, 2006). It is of interest that in spite of its relative simplicity (in terms sequence information), this technically demanding method is still considered to represent the 'gold standard' for the definition and separation of prokaryotic species (TINDALL et al., 2010). Additional approaches for prokaryotic genomic analysis include 'fingerprinting' methods based on the polymerase chain reaction such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and ARDRA, where differences in genomic "fingerprints" represent characteristic variation in genomic restriction sites, insertions and deletions (KÄMPFER; GLAESER, 2012). A more recent development has been the application of high throughput total genome sequencing techniques. These sequencing technologies are already making a big impact in the study of medically important bacteria and

fungi. In addition, the development of faster, cheaper and improved sequencing methods may serve to make genomics a tool that has a place in the workflow of a routine microbiology laboratory (SENTAUSA; FOURNIER, 2013).

Amplified ribosomal DNA restriction analysis (ARDRA) is a commonly used tool to study microbial diversity that relies on DNA polymorphism in the gene encoding the small subunit (SSU) rDNA (SU et al., 2012). The ARDRA fingerprinting method has been widely used in microbial ecological studies, including studies of food associated microbes (RODAS; FERRER; PARDO, 2003; MEDEIROS et al., 2010; SU et al., 2012). Restriction site variations have several appropriate characteristics which make them suitable for use in both the differentiation of small subunit (SSU) rDNAs between isolates and phylogenetic reconstructions (MOYER et al., 1996; STRYDOM; CAMERON; CORLI WITTHUHN, 2011). There are different types of genetic variations so-called small-scale genetic variation includes single nucleotide polymorphisms (SNPs), multi-nucleotide polymorphisms (MNPs) and microindels. MNPs are multiple, consecutive nucleotide variations of a single common length such as double nucleotide polymorphisms (DNPs) and triple nucleotide polymorphisms (TNPs) with two and three variable nucleotides, respectively. Microindels are deletions, duplications and combinations thereof involving the gain or loss of 4 to 50 nucleotides (GONZALEZ et al., 2007).

The first step in ARDRA is amplification of a fragment of the 16S rDNA containing the genetic variation; in the current study we amplified a near full length fragment of *ca* 1500 base pairs in length. This is followed by treatment of the amplified fragment with an appropriate restriction enzyme(s). Since the presence or absence of the restriction enzyme recognition site results in the formation of restriction fragments of different sizes, strain identification can be achieved by electrophoretic resolution of the fragments (MOYER et al., 1996). Restriction fragment length polymorphism (RFLP) data generated using tetrameric (i.e., having a 4-bp recognition site) restriction enzymes (TREs), although providing less direct information on the evolution of DNA sequences, are easier to obtain and more economical than complete SSU rRNA gene sequences. This is still the case today for many laboratories worldwide, even with the current advances in DNA sequencing technology. The choice of enzymes used in ARDRA analysis is essentially empirical, but it is generally agreed that a minimum of two, ideally tetrameric, enzymes should be used (MOYER et al., 1996; SKLARZ et al., 2009). More recently, the availability of freely available bioinformatics programs on the internet, e.g. NEBcutter version 2.0, has served to facilitate the process of enzyme selection by allowing *in silico* analysis of digest patterns using 16S rDNA sequences (from a range of type strains) deposited in databanks such as the NCBI GenBank.

In the present study two TREs (*Hae*III and *Hinf*1) were employed in combination with the hexameric (i.e., having a 6-bp recognition site) restriction enzyme (HRE) *Sst*I. Although not generally recommended for ARDRA studies, owing to the lower frequency of cutting sites in the genome, hexameric enzymes (HREs) can and have been used (SKLARZ et al., 2009). In the present study, the choice of enzymes was made based on those which were available in the laboratory at the time of commencing the study. Data generated with the two TREs showed that intra-group banding patterns were essentially identical and suggested that each group contained highly related species and provided general support for the phenotypic identifications. Moreover, each group of bacteria could be clearly separated from the other groups, with the notable exception of the two isolates received as *P. fluorescens* and the three strains received as *P. putida* which generated indistinguishable banding patterns when digested with the enzyme *Hinf*1.

Interestingly, digestion with *Sst*I revealed some subtle differences between the *Acinetobacter* sp. Specifically, the presence of a unique site for this enzyme in strain P162 which was absent from the amplicons derived from the other three isolates. In addition, a single site for *Sst*I was observed in the case of the two *P. fluorescens* isolates, but was absent from all *P. putida* amplicons. Taken together with the distinct patterns generated using *Hae*III, the *Sst*I, data indicated that the two groups of *Pseudomonas* spp. were indeed representatives of different species, as had been determined by phenotypic characterization. Finally, it was seen that the HRE did not cut any of the amplicons derived from the three isolates received as *A. hydrophila*, an observation which on the one hand could be considered as making a limited contribution towards differentiation of the three isolates. Yet, on the other hand, the common absence of a site for this enzyme could be interpreted as further evidence for the relatedness of the isolates.

Taken as a whole, the ARDRA data suggested that the four groups of isolates each contained highly related bacteria, but it provided no insight into which species or genera they represented. In an attempt to address this shortcoming, the banding patterns recorded in the current study were compared to those generated by *in silico* digestion of 16S rDNA sequences derived from type strains of each of the species available in the GenBank. Unfortunately, this was not possible in the case of the acinetobacters since phenotypic data had only provided genus level identifications.

In the case of *P. fluorescens*, *in silico* digestion of the type strain LMG 5167 (GenBank accession number GU198104.1) with *Hinf*I and *Sst*I generated fragments with sizes very similar to those recorded for the isolates P88 and P250 (Table 4). However, the *Hae*III digest data generated a divergent banding profile and as such did not confirm the identity of the isolates as *P. fluorescens*. In contrast, *Hae*III digestion of the 16S rDNA of *P. putida* KT2440 (GenBank accession number NC-002947.3) generated fragments of similar size to those observed with P270, P272 and P273. However, the *Hinf*I banding pattern determined for KT2440 was not consistent with those recorded for the three milk isolates (Table 4). For this reason, it was not possible to confidently ascribe species identifications to any of the pseudomonads.

Regarding the *A. hydrophila* isolates, the banding patterns generated by *in silico* digestion of the 16S rDNA from the type strain ATCC 7966 (GenBank accession number NR-043638.1) using *Hae*III and *Hinf*I were almost identical to those seen for P07, P10 and P65, strongly suggesting that all three isolates were indeed *A. hydrophila*.

The introduction of the 16S rRNA gene as molecular marker during the 1980's allowed for the first time the creation of a hierarchical taxonomic system based on one practical molecular marker (CLARRIDGE, 2004). Subsequent, comparative analyses of other molecular markers, as well as the continually expanding body of data derived from total or partial genome characterizations, have confirmed the 16S rRNA based hierarchical system as the "backbone of modern prokaryote taxonomy" (KÄMPFER; GLAESER, 2012).

In light of the inconclusive findings of our ARDRA analyses, the decision was taken to sequence the "near full length" 16S rDNA amplicons of the 12 milk derived isolates, with the goal of providing definitive identifications at the species level. The 16S rRNA gene sequence is approximately 1550 bp long and contains both conserved and hyper-variable (V1-V9) regions (CHAKRAVORTY et al., 2007). The gene is large enough and with sufficient inter-specific polymorphisms to provide differential and statistically valid measurements. Universal primers for PCR designed to anneal in the conserved regions of "all" bacterial genera are generally selected as complementary to the conserved regions at the beginning of

the gene (first 27 nucleotides) and at either the 540-bp region (500-bp product), or close to the end of the entire sequence (˜1550-bp product). The sequence of the variable regions in the amplification product is then employed to determine taxonomic relationships (ROSSELLO-MORA; AMANN, 2001; TINDALL et al., 2010). In practical terms, sequencing of a 1500-bp amplicon requires the use of a minimum of six and as many as eight sequencing primers (as used in this study) and, for reasons of economics, it is a common practice to limit sequence analysis of 16S rDNA amplicons to the first 500-bp, since for many bacterial isolates the initial 500-bp sequence provides adequate differentiation for identification (CLARRIDGE, 2004). In terms of cost, generating the 500-bp sequence is less expensive (75% reduction in cost) and easier, since it requires a minimum of only two primers. However, sometimes sequencing the entire 1500-bp region is necessary to distinguish between particular taxa or strains, including *Acinetobacter*, *Aeromonas* and *Pseudomonas*, which are considered as being taxonomically complex (JANDA; ABBOTT, 2007; NEMEC et al., 2011; BODILIS et al., 2012). Furthermore, sequencing of the entire 1500-bp sequence is also desirable and usually required when describing a new species (KÄMPFER; GLAESER, 2012).

During the last 30 years, thousands of publications have utilized 16S rRNA gene sequencing as part of a species description, or for differentiation between isolates. Analysis based on 16S rRNA gene sequences allow bacterial identification that is considered by the majority of researchers to be more robust, reproducible, and accurate than that obtained by phenotypic testing (CLARRIDGE, 2004; JANDA; ABBOTT, 2007; TINDALL et al., 2010). Moreover, because 16S rRNA gene sequence analysis can discriminate far more finely among strains of bacteria than is possible with phenotypic methods, it should, in theory, allow a more precise identification of poorly described, infrequently isolated, or so-called "phenotypically atypical" strains (KÄMPFER; GLAESER, 2012).

However, it should be noted that the use of 16S rDNA sequencing is not without limitations and caution must be exercised to avoid generating erroneous identifications. In this context, it is pertinent to discuss how 16S rDNA sequencing data should be processed and to establish what degree of sequence similarity (to type culture derived sequences) is required to arrive at a definitive identification at the species level. This is critical, since the exact homology values have a profound effect on interpretation of the outputs. In contrast to studies employing DNA hybridization, where values of >70% re-association are considered necessary to determine membership of a species, there are no defined "threshold values" (e.g. 98.5% similarity) above which there is universal agreement of what constitutes definitive and conclusive identification to the rank of species (JANDA; ABBOTT, 2007; TINDALL et al., 2010). When dealing with bacteria identified by sequencing of the 16S rRNA gene, many taxonomists accept a percentage identity score of $\geq$ 97% and $\geq$ 99% to classify a microorganism to the levels of genus and species, respectively (PETTI, 2007). This value for species was also endorsed by Janda and Abbott (2007), but according to those authors 99.5% would represent the ideal value. In practice, nucleotide sequence homology values of $\leq$ 98.7% (based on sequencing of the "full length" or "near full length gene") are commonly used to indicate membership of different species, and this value has been shown to this correlate well with DNA-DNA hybridization results (KÄMPFER; GLAESER, 2012). Yet, it should be emphasized that occasionally higher homology values may be attributed to distinct (phenotypically) species groupings (TINDALL et al., 2010). This topic continues to generate debate among microbial taxonomists. The advantages, but also pitfalls of the 16S rRNA gene sequence based studies have been thoroughly reviewed by Janda and Abbott (2007) and by

Tindall et al. (2010) and the reader is directed to those texts for a more detailed analysis of this controversial topic.

The 16S rRNA gene sequence has been determined for a very large number of strains. In this context, the NCBI GenBank (the largest databank of nucleotide sequences) held over 108 million individual sequences in 2010, of which almost 10% were represented by full length or near full length 16S rRNA gene sequences, and many more partial sequences of 500-bp or less mainly generated from metagenomics and other microbial ecological studies (BENSON et al., 2010). On the plus side, this means that there are many previously deposited sequences against which to compare the sequence of an unknown strain. Yet, on the negative side caution should be exercised when working with the data deposited in the GenBank and other open access databases. In this context, a multicenter study from the United Kingdom (ASHELFORD et al., 2005) conservatively estimated that at least 5% of the 1.399 sequences searched had substantial errors associated with them ranging from chimeras (64%) to sequencing errors or anomalies (35%). A potential solution to this problem is the use of sequences from databases which have performed screening of the sequences deposited therein. Examples of such a database are Greengenes (http://greengenes.lbl.gov) and SILVA (http://www.arb-silva.de), which provide comprehensive, quality checked and regularly updated datasets of aligned small (16S/18S, SSU) and large subunit (23S/28S, LSU) ribosomal RNA (rRNA) sequences for all three domains of life, bacteria, archaea and eukarya. However, the use of these databases is much lower than for GenBank, which reflects the fact that they hold significantly fewer sequences and because users of these sites require a higher degree of expertise in the analysis of nucleotide sequencing data than that needed to perform a BLAST search in the GenBank.

In the current study we applied sequencing of the gene encoding 16S rDNA sequencing to a total of 12 isolates. The four isolates received as *Acinetobacter* sp. (P25, P162, P164 and P165) had each been identified to the genus level by phenotypic analysis, specifically the API-20NE kit. Sequencing demonstrated that two of the isolates (P165 and P164) were *A. guillouiae*, based on their showing 99.6 % sequence homology (5 nucleotide differences over a total of 1489 base pairs), with sequences deposited as being derived from this species in the GenBank, including the type strain ATCC 11171 (GenBank accession number; HE651924.1). The isolate P162 was also identified as *A. guillouiae* ATCC 11171. Yet, this isolate showed only 97.6% homology to the type strain sequence (37 nucleotide differences over 1496 base pairs sequenced). As such, it could not be confidently identified as *A. guillouiae* employing the criteria reported by Petti (2007). It is of interest, that this strain had been identified as being different from the others based on ARDRA data (specifically the *Sst*I banding pattern). The species *A. guillouiae* has previously been reported in association with raw milk, but it has also been isolated from a number of different environments (soil, water, fish farm effluent) and from clinical material including blood, feces, sputum and urine (NEMEC et al., 2011).

The fourth *Acinetobacter* isolate (P25), which had shown identical ARDRA banding patterns to P165 and P164, demonstrated 99.4% nucleotide sequence homology (8 base pair differences over 1482 nucleotides sequences) with *A. johnsonii* type strain ATCC 17909 (Genbank accession number; HE651920.1). This finding calls into doubt the utility of the ARDRA analysis for this genus, at least using the enzymes employed in the current study. The species *A. johnsonii* has not previously been reported in association with milk, but lipolytic strains of *A. calcoaceticus* (which is virtually indistinguishable phenotypically from *A. johnsonii*) have been reported in raw milk where they have the potential to act as

psychrophilic spoilage organisms (PRATUANGDEJKUL; DHARMSTHITI, 2000). Thus, rather than representing the first report of *A. johnsonii* in association with raw milk, our sequencing data for isolate P25 could be viewed as highlighting the difficulties associated with phenotypic identification of members of this genus. According to Nemec et al. (2011), the taxonomy of the genus *Acinetobacter* has undergone extensive development over the past few decades and, currently, the genus comprises 28 species with valid names (www.bacterio.cict.fr/a/acinetobacter.html). It is noteworthy that members of this genus can form a sizeable proportion of the microbiota of raw milk, most likely reflecting their almost ubiquitous distribution in the nature. Accordingly, in the study of Gennari et al. (1992) *Acinetobacter* populations exceeded 5% of the total bacterial population in over half of the samples analyzed, and in one sample they constituted 70% of the cultivable microbiota. A total of 307 *Acinetobacter* species were identified (phenotypically) by those authors; with *A. lwoffii* representing 93% of the isolates, *A. anitratus* (5.5%) and *A. alcaligenes* (1.5%). Problems correlated with this genus in milk are linked principally to lipase production (CHRISTEN; WANG; REN, 1986; BRAUN; SUTHERLAND, 2003) and "ropy milk", a phenomenon caused by some *Acinetobacter* spp. due to the production of capsular polysaccharides.

In common with the genus *Acinetobacter*, the taxonomy of the genus *Pseudomonas* has long been considered as both complex and controversial. Attempts to classify the members of this genus using molecular based methods, including 16S rDNA sequencing, have revealed serious misidentifications based on the application of phenotypic tests (SPIERS; BUCKLING; RAINEY, 2000). Yet, diversity within the genus is not limited to physiological traits. The diversity of phenotypes is also reflected at the genetic level, and it has been suggested that the diversity of the genome architecture is of particular significance (SILBY et al., 2011). Data generated from genome fingerprinting studies have revealed a remarkable degree of restriction fragment polymorphism among strains of a species and even among strains that are indistinguishable on phenotypic grounds (HILARIO; BUCKLEY; YOUNG, 2004; SILBY et al., 2011).

In the context of the current work, it is important to stress that members of the genus *Pseudomonas* have long been recognized as the predominant group of psychrotrophic bacteria recovered from spoiled refrigerated milk (MUIR, 1996; CHEN; DANIEL; COOLBEAR, 2003). Moreover, among the pseudomonads, *P. fluorescens* is considered to be the principal spoilage agent of stored milk (MUNSCH-ALATOSSAVA and ALATOSSAVA (2006) However, the identification of the spoilage bacteria as *P. fluorescens* has frequently been based on a limited number of phenotypic tests, and as such the potential involvement of other closely related species may have been underestimated.

In the current study we received two cultures (P250 and P88) which had been identified as *P. fluorescens* based on data derived from the API-20NE system (ARCURI et al., 2008). Sequencing data indicated that the phenotypic analysis was incorrect for both isolates. This was particularly the case for P88 where the top three hits in a BLAST search were sequences deposited as being derived from isolates of *P. rhodesiae,* including the type strain CIP 104664 (GenBank accession number; NR024911.1), where a difference of only 2 nucleotides in 1455 of sequenced based pairs was recorded, resulting in a degree of sequence homology of 99.9%. In the case of isolate P250, sequence homology at the 98.7% level (19 base pair differences over 1441 nucleotides sequenced) was recorded with a sequence derived from *P. rhodesiae* (type strain CIP 104664), a sequence deposited as *P. fluorescens* (GenBank accession number; GU198125.1) and a sequence deposited as a *Pseudomonas* sp. (GenBank

accession number; JF312942.1). As such, it was not possible to conclusively identify this isolate. The species *P. rhodesiae* is closely related, phylogenetically, to *P. fluorescens*, but it has not been reported previously as a component of the microbiota of raw milk. However, we consider it likely that, as with *A. johnsonii*, the absence of evidence of an association between *P. rhodesiae* with milk is not necessarily evidence of absence. Rather, it most likely represents shortcomings in the phenotypic tests employed, coupled with the pre-formed hypothesis that the majority of fluorescent pseudomonads isolated from milk are *P. fluorescens*. Differentiation between *P. fluorescens* and its related species is not an easy task. Among the approximately 100 recognized species of *Pseudomonas*, almost 60% belong to the "so-called" *P. fluorescens* lineage, which was established based on molecular and phenotypic characterization (BODILIS et al., 2012). Sequencing of 16S rDNA was an important component of the identification process, but on its own it was not sufficient to arrive at a definite identification for a number of species within the lineage.

Pseudomonas putida has been described as an additional species of psychrophilic *Pseudomonas* involved in spoilage of cold stored milk and a variety of foodstuffs including cheese (FRANZETTI; SCARPELLINI, 2007). At the taxonomic level, this species of *Pseudomonas* lies somewhere between the *P. fluorescens* and *P. aeruginosa* lineages; exactly where will depend on which molecular markers or which phenotypic tests are employed to characterize it (BODILIS et al., 2012). Once again, the accurate identification of strains of *P. putida* is not a straightforward process.

In the current study we received three cultures (P270, P272 and P273), each identified as *P. putida* based on data from the API-20NE system (ARCURI et al., 2008). Data from our ARDRA analyses had indicated that these strains were closely related to each other and that they also shared a high degree of relatedness with the isolates received as *P. fluorescens* (P88 and P250). Sequence analysis showed the three isolates to have 99.5% intra-strain sequence homology, and submission of the sequences to the BLAST algorithm identified all three isolates as *P. psychrophila* at the level of 99% sequence homology. Yet again, these data refuted the phenotypic data and provided support for the ARDRA data which had indicated that the isolates were not *P. putida*, as determined based on a comparison with the widely studied KT2440 strain. The species *P. psychrophila* is a member of the *P. fluorescens* lineage and groups closely with *P. fragi and P. lundensis* (BODILIS et al., 2012). Both of those species have been isolated from milk and are considered to be important spoilage organisms MARCHAND et al. (2009).

The final genus of milk associated bacteria investigated in the current study was *Aeromonas*, represented by three isolates (P65, P10 and P07) all received as *A. hydrophila*, having been identified phenotypically using the API-20E and API-20NE systems (ARCURI et al., 2008). The value of using 16S rDNA sequencing for identification of members of the genus *Aeromonas* has been questioned by numerous authors, given that this sequence exhibits high similarity among *Aeromonas* species (YÁÑEZ et al., 2003; SOLER et al., 2004). Some more recent studies have investigated the usefulness of alternative housekeeping genes e.g. *gyr*B (encoding DNA gyrase) and *rpo*D (encoding the D subunit of RNA polymerase) for the identification of *Aeromonas* species, with sequence analysis of these housekeeping genes revealing higher sequence divergence than that seen for the 16S rRNA sequence among all *Aeromonas* spp. (YÁÑEZ et al., 2003; SOLER et al., 2004; KUPFER et al., 2006). Interestingly, analyses of these housekeeping genes have corrected inaccurate prevalence data of *Aeromonas* spp. that were phenotypically identified from previous clinical samples and environmental specimens (FIGUERAS et al., 2009; MARTÍNEZ-MURCIA et al., 2009;

ARAVENA-ROMÁN et al., 2011; FONTES et al., 2011; PUTHUCHEARY; PUAH; CHUA, 2012). In addition, it has become apparent that the species *A. aquariorum* may have been mistakenly identified as *A. hydrophila* or *A. caviae* due to the high similarities of the 16S rRNA sequences and biochemical profiles among these three species (ARAVENA-ROMÁN et al., 2011; FIGUERAS et al., 2011).

The three isolates examined in the current study had each presented identical banding patterns when analyzed by ARDRA, and the banding patterns were highly similar to those generated by *in silico* digestion of the 16S rDNA of a type strain of *A. hydrophila*. Taken together, these observations strongly indicated that the phenotypic identifications as *A. hydrophila* were correct. Initial sequence analysis revealed that the P07 and P10 isolates were virtually identical and showed 99.8% sequence homology (only 3 nucleotide differences over 1494 base pairs sequenced). In the case of P65, the sequence homology level was 98.8% (based on a total of 17 nucleotide differences) in comparison to P07 and P10. Submission of the sequences to BLAST searches identified P07 and P10 as *A. hydrophila* based on a level of 99.8% nucleotide homology. However the situation with the P65 isolate was more difficult to analyze. Specifically, the top hits in the BLAST searches, at the 99.8% homology level, were for sequences deposited as being derived from *A. punctata* (= *A. caviae*), but there were also sequences showing 99.7% homology which had been deposited as originating from strains of *A. hydrophila*. In an attempt to clarify this situation, we performed zymogram analysis of the extracellular proteins of the three isolates, using gelatin as the substrate. The results of these experiments, specifically the electrophoretic mobility profile of proteinases, clearly demonstrated that P65 was a different species of *Aeromonas* from P07 and P10. While the zymogram results did not conclusively identify the P65 isolate as *A. punctata*, they served to confirm that it was not *A. hydrophila*.

As stated above, the principal objective of the current study was to establish a definitive species level identification for the 12 isolates examined. At the same time, we sought to evaluate if the identification could be achieved using ARDRA analysis, or if it would be necessary to undertake the more costly option of sequencing the 16S rDNA of each isolate. It was concluded that ARDRA analysis, although relatively inexpensive and easy to undertake was unable to either confirm or refute the identifications based on phenotypic tests of the 12 isolates. As such, the value of this technique, at least with the enzymes employed in the current work, was limited. On the other hand, sequencing was able to provide confident identifications for three of the acinetobacters (above the 99% homology level), one isolate of *P. rhodesiae* (received as *P. fluorescens)*, three isolates of *P. psychrophila* (received as *P. putida)*, and two isolates of *A. hydrophila* (received as *A. hydrophila)*. In the specific case of the *Acinetobacter* isolates, sequence analysis served to improve upon the genus level identifications which had been established previously (ARCURI et al., 2008). Moreover, the use of sequence analysis with the five *Pseudomonas* isolates demonstrated that four of the five isolates had most likely been misidentified by use of phenotypic testing, despite the fact that the authors (ARCURI et al., 2008) had reported that the identities generated for the *P. fluorescens* isolates were at the level of (98.6 to 99.9%) and at a value of 99.1% for the *P. putida* isolates. Moreover, the identification of the P250 isolate was not resolved satisfactorily by sequencing of the 16S rRNA gene.

The inability to effectively identify members of the genus *Aeromonas* using a limited number of phenotypic tests is well recognized (JANDA; ABBOTT, 2010). This difficulty was reflected by the observation that the accuracy reported for the API-20 system to identify *Aeromonas* species was lower (92.9 to 99.9%) than for the pseudomonads (ARCURI et al.,

2008). Furthermore, sequencing coupled with zymogram analysis demonstrated that the P65 isolate had been misidentified as *A. hydrophila*, and was most likely a strain of *A. caviae*. Interestingly, a single isolate identified as *A. caviae* (at the level of 98.9%) was reported in the study of Arcuri et al. (2008).

5 CONCLUSION

Bacterial species identification requires a stable and overall accepted classification. Such a classification is still elusive for a large number of prokaryotic species. With a few notable exceptions, there are no short-cuts to arriving at a precise identification of a bacterium. Polyphasic characterization integrates both phenotypic and genotypic traits and the term 'polyphasic' was first introduced by Colwell (1970) who used the term *polyphasic taxonomy* to refer to classifications based on a consensus of all available methods: single character tests, as well as multiple-character tests, including both phenotypic and genotypic (genomic) data. It is becoming increasing obvious that only the combination of data generated by genetic and phenotypic testing can provide a firm structure for elucidating the diversity of prokaryotes (TINDALL et al., 2010; KÄMPFER; GLAESER, 2012).

Our data indicated that neither phenotypic nor genotypic methods were sufficient to confidently identify some of isolates included in this study. The inclusion of an addition molecular marker would most likely have helped to resolve the difficulties encountered, but at the same time it would have introduced an unacceptable level of cost. An alternative, as used in this study with the *Aeromonas* isolates, was to employ an additional molecular/phenotypic method (electrophoretic separation of proteins coupled to an assessment of proteolytic activities). The data generated from this approach was not enough to conclusively identify the isolates, but it did serve to reinforce the differences detected by sequencing and confirmed the misidentification of isolate P65 as *A. hydrophila*.

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CAPÍTULO IV

MILK FRAUD BY WHEY ADDITION: NEW POTENTIAL PROTEOLYTIC MICROBIAL PLAYERS HINDERING ITS DETECTION

ABSTRACT

Problems related to the quality of milk and dairy products due to psychrotrophic growth are compounded by the fact that their proteolytic activity mask results of fraud detection by whey addition in tests based on CMP index. The aim of this research was to characterize some psychrotrophic bacteria in relation to this interfering potential. The population growth dynamics as well as proteases and caseinmacropeptide (CMP) production by 12 strains of psychrotrophic bacteria isolated from raw milk was assessed. A population of *ca* 10^3 CFU mL^{-1} of each isolate was inoculated separately in thermally treated milk (121°C/3min) and then split into seven sub-samples each incubated at different refrigeration temperature and time, following full experimental design (2^2) with three repetitions in the central point. According to the results of HPLC-GF (Brazilian official method) and immunochromatographic tests, with the exception of the four acinetobacters, all strains were capable of producing CMP, most notably following incubation at 10°C. The production of pseudo CMP, which is characteristic of the bacterial proteolysis of casein, was evaluated by HPLC-ESI-MS/MS and, except for one strain (*Aeromonas punctata*), all isolates produced more than 50% of pseudo CMP concentration when compared with true CMP. This observation suggests that, at present, HPLC-ESI-MS/MS is the only method capable of differentiating between fraud by whey addition and poor microbiological quality in milk. Such differentiation is necessary to assure the correct punishment is applied to the fraudster.

Keywords: psychrotrophic, caseinmacropeptide, HPLC-ESI-MS/MS

RESUMO

Problemas relacionados com a qualidade do leite e produtos lácteos devido ao crescimento de bactérias psicrotróficas são agravados pelo fato de que sua atividade proteolítica mascara resultados de detecção de fraude por adição de soro em testes baseados no índice de CMP. O objetivo desta pesquisa foi caracterizar algumas bactérias psicrotróficas em relação a este potencial interferente. A dinâmica de crescimento populacional, assim como a produção de proteases e caseinomacropeptídeo (CMP) por 12 linhagens de bactérias psicrotróficas isoladas de leite cru foram avaliadas. Uma população de *ca* 10^3 UFC mL^{-1} de cada linhagem foi inoculada separadamente no leite tratado termicamente (121°C/3min) e, em seguida, dividido em sete subamostras, cada uma incubada em diferentes tempos e temperaturas de refrigeração, de acordo delineamento experimental completo (2^2) com três repetições no ponto central. De acordo com os resultados da HPLC-FG (método Brasileiro oficial) e de teste imunocromatográfico, com exceção das quatro linhagens de acinetobacters, todas as linhagens foram capazes de produzir CMP, mais notavelmente após incubação a 10°C. A produção de pseudo CMP, que é característica da proteólise bacteriana de caseína, foi avaliada por HPLC-ESI-MS/MS e, com a exceção de uma linhagem (*Aeromonas punctata*), todas as demais apresentam mais de 50% da concentração de pseudo CMP quando comparado com CMP

verdadeiro. Esta observação sugere que, atualmente, HPLC-ESI-MS/MS é o único método capaz de diferenciar entre fraude por adição de soro e baixa qualidade microbiológica do leite. Esta diferenciação é necessária para assegurar que a correta punição é aplicada ao fraudador.

Palavras-chave: psicrotróficos, caseinomacropeptídeo, HPLC-ESI-MS/MS

1 INTRODUCTION

The authenticity of dairy products has become a focal point, attracting the attention of scientists, producers, consumers, and policymakers (DE LA FUENTE; JUÁREZ, 2005). When an authentic food product is substituted in whole or in part with a less expensive substitute, it is food fraud whenever the purchaser is not aware of this, characterizing the intention to deceive the purchaser in order to increase economic gain for the seller (HERMANS, 2012).

Milk and its products (ice cream, yogurt, butter, cheese, etc.) are in considerable demand, commanding premium prices and are, therefore, vulnerable to economic adulteration (KAROUI; DE BAERDEMAEKER, 2007). Indeed, with an extensive collection and information analysis of food ingredient frauds and economically motivated adulterations from 1980 to 2010, Moore et al. (2012), based on scholarly reports dataset, concluded that from the 25 ingredients examined, milk occupied the second position (14%) among the food ingredients with the greatest number of records or hits leaving the first position to oils.

In practice, the most common frauds in milk and milk products are substitution of part of the fat or proteins, blends of milk coming from different species, the addition of low-cost dairy products, or mislabeling of products protected by denomination of origin (DE LA FUENTE; JUÁREZ, 2005). In some countries, milk is subject to adulteration by addition of whey (FURTADO et al., 2011). In Brazil, besides whey, water and butter milk are also commonly added to the milk (VELLOSO, 2003).

Besides deceiving the purchaser, frauds involving additions of low-cost dairy products may result in a decrease of the nutritional value of milk and or can cause other problems such as the introduction of harmful bacteria and/or chemical substances. When the fraud is committed by milk farmers, it also poses a decrease of yield for the cheese industry.

Traditionally, the liquid whey was considered as an essentially valueless by-product in the manufacturing of cheese and casein. As such, it was generally treated as sewage by the industry or, in some cases, used as a feed for pigs (BALAGTAS et al., 2003; MANSO; LÓPEZ-FANDIÑO, 2004). It should be noted that, owing to its high biochemical and chemical oxygen demand, the cost associated with the correct disposal of whey outweighed its economic values which resulted in many producers simply discharging it, untreated into rivers. However, nowadays, liquid whey has gained the status of a co-product, because of the many possibilities it offers as a food ingredient (MANSO; LÓPEZ-FANDIÑO, 2004) and also owing to its functional properties (DEL MAR CONTRERAS et al., 2008; NEELIMA et al., 2013).

Nevertheless, it seems clear that although there now several ways of employing whey, increased production driven by the growth of cheese and industrial caseinate production, coupled with high disposal costs, have combined to make the fraudulently addition of whey to dairy products financially attractive (RECIO et al., 2000b; DE LA FUENTE; JUÁREZ, 2005). Moreover, another reason, particular to the European Union, which makes this practice attractive is the provision of a public subsidy for the storage of skimmed milk powder and the processing of these powders for use as animal feed (BREMER et al., 2008).

Various methods for detection of whey in milk have been reported and they can be categorized in two groups: a) techniques based on detecting the presence of glycomacropeptide (GMP) in the suspected samples (GMP-based methods); b) based on the compositional difference between cheese/rennet whey and milk (non-GMP-based methods)

(NEELIMA et al., 2013). In general, the regulatory authorities of countries where this type of fraud is common recommend GMP-based methods method for the detection of milk adulteration by whey addition (FURTADO et al., 2011).

GMP (also called CMP in a non-glycosylated form) is the hydrophilic portion from aminoacid 106 to 169 of κ-casein (THOMÄ; KRAUSE; KULOZIK, 2006). This molecule is present in whey because during cheese production rennet cleaves κ-casein into GMP and para-κ-casein (from aminoacid 1 to 105), thereby destabilizing the casein micelle and resulting in aggregation and gel formation of the micelles (VASBINDER; ROLLEMA; DE KRUIF, 2003).

However, it has been observed that proteolytic enzymes produced by some psychrotrophic bacteria can cause false-positive results in the majority of GMP-based methods, given that they cleave the κ-casein molecule at the same position as enzymes from rennet, producing peptides like CMP (RECIO et al., 2000a; BREMER et al., 2008; FURTADO et al., 2011). This situation leads to inconclusive results and, consequently, the proper correct legal penalty cannot be applied to the fraudster, who may instead receive a milder penalty for poor microbiological quality.

The occurrence of false-positive results as a consequence of bacterial proteolysis has highlighted *Pseudomonas fluorescens* as the principal causative agent (RECIO et al., 1996; RECIO et al., 2000b; MOTTA; HOFF, 2009; OLIVEIRA et al., 2009), most likely owing to the predominance of this species in the microbiota of cooled raw milk prior to processing. However, other species of psychrotrophic proteases producers may be present in the milk microbiota and only a limited number of studies have attempted to characterize specific microorganisms and their respective enzymes (TONDO et al., 2004; ARCURI et al., 2008; NÖRNBERG et al., 2010; RASOLOFO et al., 2010).

In the current study, a collection (n=12) of psychrotrophic bacteria previously isolated from raw milk by researchers at the Brazilian Agricultural Research Corporation (EMBRAPA), and phenotypically identified as *Acinetobacter* sp. (n=4), *Aeromonas hydrophila* (n=3), *Pseudomonas fluorescens* (n=2) and *Pseudomonas putida* (n=3) (ARCURI et al., 2008) were evaluated for the ability to grow in milk, produce proteases and, consequently, CMP and/or similar products. The results of the different microbial proteolysis activities were compared using available Brazilian official methods as well as a rapid ELISA screening test.

2 MATERIAL AND METHODS

2.1 Milk

Freshly collected raw milk samples (first jet) were obtained directly from a milking machine pipe into a sanitized gallon, then skimmed (<0.5% fat) and thermally treated at 121°C/3min (LEWIS, 2003) in 200 mL glass bottles (Figure 5).

Figure 5. a) Milk samples being a) collected and b) skimmed; c) glass bottles with skimmed milk (left) and fat (right).

2.2 Strains Selection Criteria and Growth Conditions

Twelve psychrotrophic strains isolated from raw milk were obtained from microbial network of the National Platform of Genetic Resources (CA5) at the Brazilian Agricultural Research Corporation (EMBRAPA) - Dairy Cattle division (Juiz de Fora, MG, Brazil). The main selection criterion was their ability to produce a proteolysis halo in milk agar (MA) at temperatures of 4, 7 and/or 10°C, as had been previously tested by EMBRAPA (ARCURI et al., 2008). The stock cultures were kept frozen at -25°C in tryptone soya broth (Oxoid, Basingstoke, UK) supplemented with 20% (vol/vol) glycerol and refrigerated at 4°C on nutrient agar inclined, being transferred to fresh maintenance medium every 4 months. At the time of use, the cultures were transferred to tryptone soya broth and incubated for 24h/30°C. This procedure was repeated an additional two times consecutively.

2.3 Identity of the Strains

The microorganisms employed had been previously identified phenotypically (API 20E and API 20NE, BioMérieux) by researchers at EMBRAPA (ARCURI et al., 2008) as *Acinetobacter* sp. (P25, P162, P164 and P165), *Aeromonas hydrophila* (P07, P10 and P65), *Pseudomonas fluorescens* (P88 and P250) and *Pseudomonas putida* (P270, P272 and P273). The identity of the strains was examined by 16S rDNA sequencing following the methodology described previously (Chapter II). The sequences obtained were compared to the

GenBank databases using the BLAST algorithm. The identification derived via phenotypic and genotypic methods matched for all strains at the genus level. However, as shows in chapter II, genotypic analysis refuted the majority of the phenotypic identification at the species level. As such, the strains will be referred to in this work, according to the identification derived from 16S rDNA sequencing, as *Acinetobacter johnsonii* (P25), *Acinetobacter guillouiae* (P162, P164 and P165), *Aeromonas hydrophila* (P07 and P10), *Aeromonas punctata* (P65), *Pseudomonas rhodesiae* (P88 and P250), *Pseudomonas psychrophila* (P270, P272 and P273).

2.4 Proteolytic Activity in Milk Agar

Milk agar was prepared by adding to nutrient agar (Himedia, Mumbai, India) 10% of skimmed milk (Molico®, Nestlé) which was previously reconstituted (100 g L^{-1}) and sterilized at 110°C/10min. The next steps were performed according to Arcuri et al. (2008). Each inoculum of psychrotrophic bacteria was freshly prepared (one colony diluted according to the Mac Farland scale 0.5 in saline - NaCl w/v 0.85%) and 1 µl of this suspension then spread with a sterile loop on the surface of the plates. The plates were incubated at 4, 7 and 10°C and evaluated for up to 10 days for the formation of transparent halos around the colonies, which was considered indicative of proteolysis. The size of the halo radius was also measured and recorded. In order to check the possibility of so-called "pseudo proteolysis", the culture medium was covered with a solution of 10% acetic acid for 1 min, following after the 10 day incubation period. The excess liquid was removed, and the maintenance of the halo was confirmed.

2.5 Inoculum Standardization

In order to know the exact concentration of the inoculum, the linear regression equation to transform absorbance data in CFU mL^{-1} was determined for each culture. Thus, beginning with the cell suspension obtained from the third culture in tryptone soya broth dilutions 1:5, 1:10, 1:25 and 1:50 were prepared in distilled water and the absorbance value at 600 nm was read for each dilution. Concurrently, quantification of the number of viable cells was performed by plating aliquots of 0.1 mL of serial decimal dilutions of cultures, prepared in peptone water (0.1% w/v), on the surface of plate count agar (Himedia). The plates were incubated at 30°C during 48h. These values were correlated and employed to plot graphs of absorbance x CFU mL^{-1} to determine the equation through simple linear regression.

2.6 Experimental Design and Microbial Population Counting

Full factorial design associated with response surface analysis, with three central points was used as an experimental design. The matrix with their coded and real levels (in parentheses) is showed in the Table 5 for each variable studied. The real levels chosen were based on existing Brazilian rules for milk storage as well as over conditions (e.g. 10°C/4d), necessary for the matrix construction.

Table 5. The matrix design of the tests generated by full factorial design (2^2), with their coded and real levels (in parentheses).

Test	Time (days)	Temperature (°C)
1	-1 (2)	-1 (4)
2	+1 (4)	-1 (4)
3	-1 (2)	+1 (10)
4	+1 (4)	+1 (10)
5	0 (3)	0 (7)
6	0 (3)	0 (7)
7	0 (3)	0 (7)

Inoculated milk (1 x 10^3 CFU mL^{-1}) was incubated at times and temperatures specified in the matrix design. At the end of each period, the microbial population for each sample was enumerated by plating aliquots of 0.1 mL of serial decimal dilutions of cultures, prepared in 0.1% peptone water, on the surface of plate count agar (Himedia) with the incubation at 30°C during 48h. In addition, an aliquot (40 mL) of the sample was transferred to a vial containing a tablet of Broad Spectrum Microtabs® II (D&F Control Systems, Inc. Massachusetts, USA) and frozen until subsequent analysis. An aliquot of the milk was left uninoculated and served as a blank/negative control.

2.7 Challenge Testing at 30°C

At the end of the incubation period, shown in Table 5, an aliquot of each sample was once again thermally treated (121°C/3min) and incubated up to 120 days at 30°C for observation of the characteristics of the enzyme activities such as apparent proteolysis, and presence of sediments, clots and/or clumps, among others.

2.8 CMP Analysis

2.8.1 HPLC-GF

All analyses were performed at Laboratório de Análise da Qualidade do Leite (LabUFMG, UFMG, Belo Horizonte, MG, Brazil) following the official methodology (BRASIL, 2010).

All samples were transported frozen to the laboratory and were unfrozen in a water bath at 30°C. Following homogenization, 5 ml of 24% trichloroacetic acid (TCA) was added to individual aliquots of 10 mL drop by drop and under conditions of constant stirring. After standing for 60 min at room temperature, the samples were filtered through in qualitative filter paper (Whatman™ nº 4, GE Healthcare, Buckinghamshire, UK) and then injected into the liquid chromatograph (Shimadzu, Kyoto, Japan).

The mobile phase was comprised by a pH 6 phosphate buffer solution (1.74 g potassium hydrogen phosphate; 12.37 g of potassium dihydrogen phosphate and 21.41 g of sodium sulfate in approximately 700 mL of distilled deionized water) with the pH adjusted to 6 using phosphoric acid solution 3 mol L^{-1} and potassium hydroxide solution 3 mol L^{-1}. Then,

the solution was transferred to a 1000 mL volumetric flask and the volume was completed with distilled deionized water and filtered through a membrane of 0.45μm. Before use, the solution was degassed in an ultrasound bath for 15 min.

The calibration curve of CMP was prepared in a matrix of reconstituted skim milk powder (100 g L^{-1}) (Difco, Sparks, MD, USA) comprising 15 mg L^{-1}, 30 mg L^{-1} 45 mg L^{-1} 60 mg L^{-1} 75 mg L^{-1} and 90 mg L^{-1}, and a blank (0 mg L^{-1}). A graph of concentration (mg L^{-1}) *versus* the effective height of the peak was constructed and then the linear regression curve, accepting R values > 0.95.

Peaks with the same retention time as standard CMP were identified and the concentrations presented in the samples were calculated as mg L^{-1}, to within one decimal place, based on the calibration curve.

2.8.2 Immunoassay test

Sixteen samples from different strains of the experimental design (Table 5) were selected to perform the immunoassay test (Stick c-GMP, Cap Lab, São Paulo, Brazil). The experimental conditions to which those samples had been subjected were 4°C and 10°C for 2 days. Analyses were carried out according to the manufacturer's instructions by adding 4 mL of 20% TCA to 6 mL of the milk sample (in a test tube with screw cap). After homogenization in an automatic shaker, the contents of the tube were left standing for 10 min and then centrifuged at 1500 rpm for 10 min. The supernatant was filtered on qualitative filter paper (Whatman™ n° 4) and the filtrate diluted 1000 times in buffer solution provided by the manufacturer. A volume of 500 μl of this dilution was transferred to an empty tube, which was inserted into the Stick c-GMP, which remained in contact with the sample for 5 min. After this period, the tip was cut off and reading was performed. The presence of the red color ribbons indicated positivity for the presence of C-GMP, and the intensity was compared to a reference card, where it was proportional to the concentration of CMP (0, 30 and 75 mg L^{-1}) in the sample.

2.8.3 HPLC-ESI/MS/MS

These analyses were performed at Laboratório Nacional Agropecuário (LANAGRO, Porto Alegre, RS, Brazil) following the official methodology (BRASIL, 2010).

All samples were transported frozen to the laboratory and were unfrozen in a water bath at 30°C. After homogenization, in each aliquot of 10 mL was added 5 mL of 24% TCA. The samples were kept for 20 min in an ultrasound bath (40 kHz) and then filtered in qualitative filter paper (Whatman™ n° 4).

The CMP was extracted by Solid-phase Extraction (SPE), according to Motta et al. (2011). The tryptic digestion was performed with trypsin (Fluka, Buchs, Switzerland) reconstituted in Ammonium bicarbonate - (NH4)HCO3 50 mM (enzyme substrate ratio 50:1) by incubation of sealed tubes under stirring at 37°C during 4 h.

The samples were injected in a HPLC coupled to a triple quadrupole mass spectrometry (HPLC-ESI-MS/MS) system in tandem mode consisting of an Agilent 1100 HPLC system (Agilent, Santa Claire, CA, USA) and an API 5000 (AB Sciex, Foster City, CA, USA).

HPLC separation was accomplished using a PLRP-S column (model P/N 1512-3401, Varian, Palo Alto, CA, USA), with gradients of acetonitrile (Merck, Darmstadt, Germany) and water, both added 0.1% of acetic acid and 0.02% of trifluoroacetic acid (TFA).

Samples were ionized by ESI in the positive ion mode. Analysis was based in N-terminal fragment MAIPPKK for CMP and AIPPK for pseudo CMP. Six fragments released from CMP tryptic digestion with good ionization intensity were monitored, covering the peptides $[M+2H]2^+$ and for MAIPPKK and $[M+2H]2^+$ for AIPPK. Two of them were used as quantifiers and four of them as qualifiers.

The working standard solutions of CMP (Davisco, Eden Prairie, MN, USA) were prepared in deionized water (1 mg mL^{-1}) and digestion tryptic curve was performed in order to check the linearity. For the optimization of ion transfer and fragmentation parameters in mass spectrometer and chromatographic separation, synthetic standards of peptides MAIPPKK and AIPPKK (Proteimax, Cotia, SP, Brasil) were used.

Curves of the natural MAIPPKK and AIPPK peptides were prepared to check the CMP concentration. The CMP content was also determined using peak area as the means of measurement.

2.9 Quantification of Extracellular Proteolytic Activity

Initially, the milk samples originating from the full factorial design (Table 5) were sorted in descending order according to the final population for each strain. After this, the extracellular proteolytic activity was tested starting with the highest population until the last population capable of generating reliable spectrophotometric reading. The method used was adapted from Bendicho et al. (2002). 500 ul of 1% azocasein solution in phosphate buffer: 36 mL of dibasic sodium phosphate (Na$_2$HPO$_4$) + 14 mL 0.05 M potassium phosphate monobasic (KH$_2$PO$_4$) 0.05 M adjusted to 1000 mL and pH 7.2, were added to 50 µl of the sample containing enzyme (milk samples). The reaction took place during 180 min at 36°C and 400 rpm on an Eppendorf Comfort mixer$^®$ apparatus. After this period, the reaction was stopped by addition of 1000 µl of TCA (5%) and the tubes were placed in an ice bath for 10 min. Thereafter, the tubes were centrifuged at 1200 x g for 10 min. Finally, 800 µl of the supernatant was added to 250 µl of 1.8 N NaOH mixed by inversion and read spectrophotometrically at 420 nm. All measurements were performed in duplicate and one unit of activity was defined as the amount of enzyme required to produce an increase in absorbance at 420 nm of 0.01 h^{-1}.

2.10 Statistical Analysis

The dependents variables from full factorial design were analyzed by Statistica 7.0 (Statsoft Inc.).

3 RESULTS

3.1 Proteolytic Activity in Milk Agar

All *Pseudomonas* spp. were proteolytic at all temperatures, while *Acinetobacter* spp. were not at any of the three temperatures. *Aeromonas* spp. showed proteolytic activity at 7ºC and 10ºC, but not at 4ºC (Table 6). Based on the size of the halos, *P. rhodesiae* were considered the most proteolytic species followed by *P. psychrophila*.

When compared to the previous report, the proteolytic behavior of the *Acinetobacter* strains was different. Thus, in the present study, proteolytic activity by *Acinetobacter* spp. was not detected at any temperature (Table 6) while it had been detected at 7ºC and 10ºC by Arcuri et al. (2008). At the same time, these authors also reported proteolytic activity of *A. punctata* at 4°C, while it was not observed in this study as well as the non-colony development at this temperature for all *Aeromonas* spp. It is considered possible that differences in the culture medium or the loss of proteolytic capacity of the cultures after successive subculturing can explain the divergent results. The lack of proteolytic activity can be observed in Figure 6.

Table 6. Proteolytic activity in milk agar of different strains of psychrotrophic bacteria and radius of inhibition zone (mm) after 10 days.

| Strain	Temperature		
	4°C	7°C	10°C
Aeromonas hydrophila			
P07	N	Y (12)	Y (12)
P10	N	Y (10)	Y (9)
Aeromonas punctata			
P65	N	Y (9)	Y (9)
Acinetobacter johnsonii			
P25	N	N	N
Acinetobacter guillouiae			
P162	N	N	N
P164	N	N	N
P165	N	N	N
Pseudomonas rhodesiae			
P88	Y (8)	Y (17)	Y (16)
P250	Y (11)	Y (16)	Y (16)
Pseudomonas psychrophila			
P272	Y (3)	Y (4)	Y (7)
P270	Y (9)	Y (14)	Y (14)
P273	Y (1)	Y (2)	Y (3)
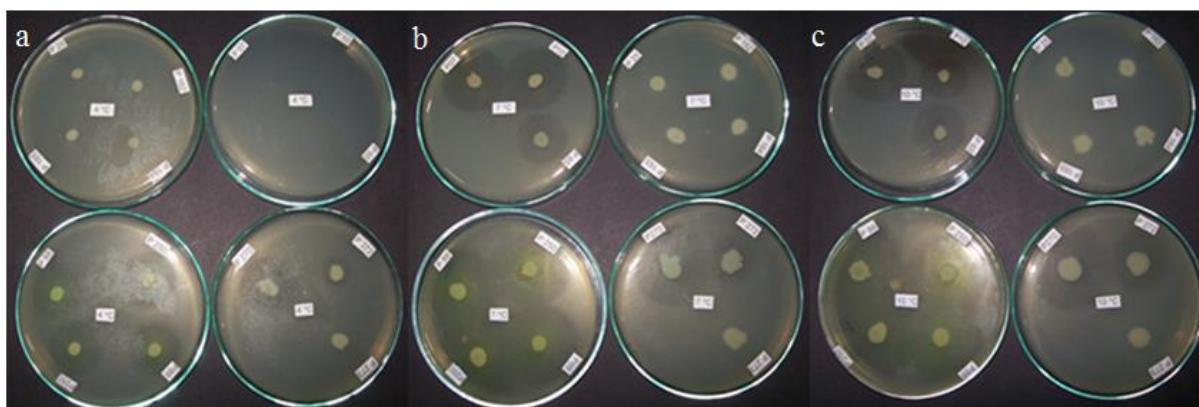
Figure 6. Proteolytic activity in milk agar after 10 days at a) 4°C; b) 7°C and c) 10°C. Clockwise direction: a) *Acinetobacter* spp., *Aeromonas* spp., *P. psychrophila* and *P. rhodesiae*; b) *Aeromonas* spp., *Acinetobacter* spp., *P. psychrophila* and *P. rhodesiae*; c) = b).

3.2 Psychrotrophic Bacterial Counts and CMP Production

The conditions that minimize and maximize psychrotrophic bacterial growth were, respectively, the combination of the independent variables 4°C/2d and 10°C/4d (Table 7). Consequently, the bacteria generation times (GT) were also higher in the condition 4°C/2d, while the strain P07 of *A. hydrophila* showed a slight population reduction.

Similarly, production of CMP by strains of *A. hydrophila* (P07, P10), *A. punctata* (P65), *P. rhodesiae* (P88 and P250), *P. psychrophila* (P270, P272, P273) were also maximized by the combination of independent variables 10°C/4 d, while it was indifferent for *Acinetobacter* spp. strains (P162, P164, P165 and P25), despite the highest population growth observed for P162 strain when growing at 10ºC for 4 days (Table 7). On the other hand, the combination of independent variables which minimizes the CMP was strain dependent, as such it did not allow inference about the variables studied (Table 7).

Table 7. Psychrotrophic bacterial counts and CMP production determined by HPLC-GF, according to experimental design… (continued on the next pages).

Strain	Inoculum (log CFU mL^{-1})	T (°C)	t (days)	FP (log CFU mL^{-1})	GT (min)	CMP (mg L^{-1})
A. hydrophila P07	3.25	4	2	3.07	-	13.35
		4	4	3.44	9105	12.27
		10	2	6.85	241	276.30
		10	4	7.94	370	1239.00
		7	3	4.95	764	5.11
		7	3	4.68	908	8.32
		7	3	4.75	867	7.44
A. hydrophila P10	3.48	4	2	3.51	24937	18.69
		4	4	3.94	3763	12.27
		10	2	6.95	250	277.16

Table 7. Continuation.

Strain	Inoculum (log CFU mL^{-1})	T (°C)	t (days)	FP (log CFU mL^{-1})	GT (min)	CMP (mg L^{-1})
A. hydrophila P10	3.48	10	4	8.91	319	1221.36
		7	3	6.79	392	11.21
		7	3	6.38	448	14.36
		7	3	6.95	374	13.42
A. punctata P65	3.73	4	2	4.00	3279	10.30
		4	4	6.04	751	6.01
		10	2	7.18	251	11.17
		10	4	8.05	402	988.87
		7	3	6.28	510	9.50
		7	3	6.23	520	9.61
		7	3	5.79	632	5.24
P. rhodesiae P88	3.00	4	2	3.96	901	10.28
		4	4	5.93	592	12.64
		10	2	7.38	198	26.95
		10	4	8.52	314	1289.44
		7	3	7.56	285	14.90
		7	3	7.49	290	13.12
		7	3	7.37	297	10.24
P. rhodesiae P250	3.36	4	2	3.93	1522	10.87
		4	4	6.05	643	5.46
		10	2	7.62	203	369.86
		10	4	8.41	344	1342.43
		7	3	7.26	333	6.78
		7	3	7.23	336	7.50
		7	3	7.37	324	9.00
A. guillouiae P162	3.41	4	2	3.60	4634	10.06
		4	4	4.18	2264	8.68
		10	2	7.34	221	12.22
		10	4	9.64	279	10.18
		7	3	6.83	380	7.68
		7	3	7.17	347	7.33
		7	3	6.94	369	7.51
A. guillouiae P164	3.40	4	2	3.74	2532	17.90
		4	4	5.16	983	18.09
		10	2	7.23	226	17.80
		10	4	8.17	363	18.14
		7	3	7.36	329	18.87

Table 7. Continuation.

Strain	Inoculum (log CFU mL^{-1})	T (°C)	t (days)	FP (log CFU mL^{-1})	GT (min)	CMP (mg L^{-1})
A. guillouiae P164	3.4	7	3	7.46	320	17.84
		7	3	7.45	321	19.07
A. guillouiae P165	3.08	4	2	3.64	1555	19.14
		4	4	5.12	850	18.33
		10	2	7.48	197	21.38
		10	4	7.98	354	27.79
		7	3	7.57	289	19.66
		7	3	7.42	300	19.34
		7	3	7.17	318	17.97
A. johnsonii P25	3.48	4	2	4.05	1519	7.98
		4	4	4.92	1201	7.08
		10	2	7.28	228	7.88
		10	4	8.28	361	7.59
		7	3	7.40	332	8.24
		7	3	7.48	325	7.85
		7	3	7.41	331	8.03
P. psychrophila P270	3.90	4	2	5.29	624	11.32
		4	4	7.38	498	9.26
		10	2	7.73	227	134.68
		10	4	8.54	373	895.75
		7	3	7.41	371	16.73
		7	3	7.54	357	11.13
		7	3	7.47	364	12.36
P. psychrophila P272	3.30	4	2	4.62	659	13.28
		4	4	6.87	486	7.59
		10	2	7.54	205	32.55
		10	4	8.50	333	798.99
		7	3	7.54	307	47.17
		7	3	7.59	303	47.93
		7	3	7.43	315	48.68
P. psychrophila P273	3.26	4	2	4.51	691	17.85
		4	4	7.26	433	16.72
		10	2	7.43	208	31.28
		10	4	9.01	301	868.93
		7	3	7.96	276	28.88
		7	3	8.21	263	28.42
		7	3	8.01	273	27.96

Inoculum= initial population; T= temperature; t= time; FP= final population; GT= generation time (GT= 60 min x h/ {(log N- log N_0)/0.301}; CMP= caseinmacropeptide

The proteolytic activity as measured through CMP detection by HPLC-GF was in accordance with the results obtained in milk agar. Thus, the levels of CMP produced by *Acinetobacter* spp. at all incubation conditions were below the levels accepted by Brazilian law (BRASIL, 2006) and not detected in milk agar.

The parameters adopted for the construction of models and based on Rodrigues and Iemma (2005) were: independent variables with significant regression coefficients at 90% or 95% of confidence limit; good repeatability at the central point; calculated F value (Fcal) greater than critical F value (Fcrit); and good percentage of variance explained by the model (R-squared or R-sqr).

The population growth regression coefficient was significant at a confidence limit of 95% for all psychrotrophics, except for the isolate P273 (with 90%), when the linear terms of the independent variable temperature (T) were considered, and less frequent for variable time (t) and interactions between temperature and time (T x t) (Table 8). The terms no incorporated into the models were included in the residue of ANOVA. Fcal was significant for all strains. There was a good repeatability in the central point and the percentage of variance explained by the model ranged between 0.794 and 0.982. The models presented, in general, fitted well with the experimental data. All cultures showed the same trend. Representative graphs of the highest and the lowest population are presented (Figure 7 and Figure 8).

Table 8. Models for the dependent variable population growing of psychrotrophic bacteria.

Strain	Model	R-sqr	C. Limit	Fcal	Fcrit
P07	5.10 + 2.07 x T	0.972	95%	72.31	6.61
P10	6.20 + 2.10 x T	0.929	95%	25.05	6.61
P65	6.23 + 0.73 x t + 1.30 x T	0.976	95%	30.87	6.94
P88	6.89 + 1.50 x T	0.865	95%	10.29	6.61
P250	6.84 + 1.51 x T	0.917	95%	12.59	6.61
P162	6.53 + 2.30 x T	0.955	95%	26.85	6.61
P164	6.65 + 1.62 x T	0.794	95%	11.56	6.61
P165	6.62 + 1.68 x T	0.799	95%	12.89	6.61
P25	6.69 + 1.65 x T	0.803	95%	14.42	6.61
P270	7.34 + 0.73 t + 0.90 x T - 0.32 x t x T	0.982	95%	54.17	9.28
P272	7.15 + 0.80 x t + 1.14 x T	0.920	95%	13.79	6.94
P273	7.48 + 1.08 x t + 1.17 x T	0.854	90%	10.01	4.33

t = time; T = temperature; C. limit = confidence limit; Fcal = calculated F value; Fcrit = critical F value

P162: Fitted Surface; Variable: Microbial population (Log CFU mL⁻¹)
2**(2-0) design; MS Residual=.3757313
DV: Microbial population (Log CFU mL⁻¹)
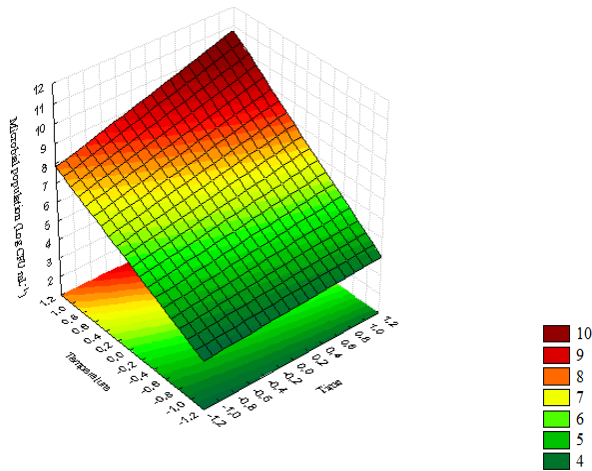
Figure 7. *Acinetobacter guillouiae* P162 population curve representing the highest population observed.

P07: Fitted Surface; Variable: Microbial population (Log CFU mL⁻¹)
2**(2-0) design; MS Residual=.1715794
DV: Microbial population (Log CFU mL⁻¹)
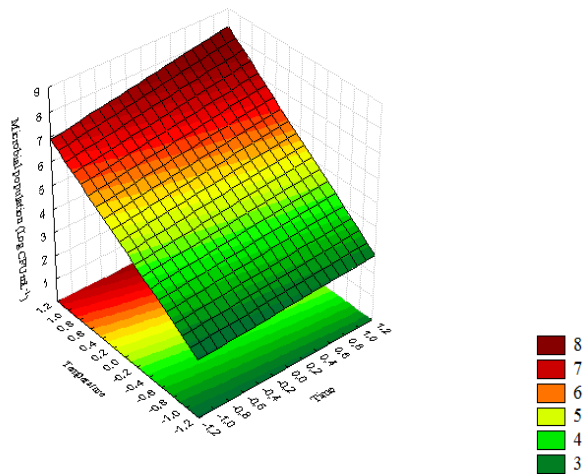
Figure 8. *Aeromonas hydrophila* P07 population curve representing the lowest population observed.

The independent variable temperature was present in 100% of the models developed from the experimental design and as the exclusive influencing variable in 66.7%, confirming that it was the most important factor contributing to the psychrotrophic population growth in this study.

However, when the dependent variable was CMP production the dataset of 50% of the strains (P07, P10, P88, P250, P162 and P270) presented significant regression coefficients only after the curvature check, which indicated a quadratic (or nonlinear) behavior. In this case, representative statistical analyses and curves are shown in Figure 9 and Figure 10. Even

with the application of the curvature check, two strains (P25 and P164) did not show significant regression coefficients. Models were created, with 90% confidence limit, for only four strains (P65, P165, P272 and P273) (Table 9).

a)

	P07; Regr. Coef; Var.:Caseinmacropeptide (mg/mL) 2**(2-0) design; MS Residual=81767.96; Adj:.6117 DV: Caseinmacropeptide (mg/mL); R-sqr=.80585					
Factor	Regressn Coeff.	Std.Err.	t(3)	p	-95.% Cnf.Limt	+95.% Cnf.Limt
Mean/Interc.	223.11	108.08	2.06	0.1309	-120.84	567.07
(1)Time	240.40	142.98	1.68	0.1913	-214.61	695.42
(2)Temperature	372.42	142.98	2.60	0.0800	-82.59	827.43
1 by 2	240.94	142.98	1.69	0.1905	-214.07	695.96

b)

	P07; Regr. Coef.; Var.:Caseinmacropeptide (mg/mL) 2**(2-0) design; MS Residual=81767.96; Adj:.6117 DV: Caseinmacropeptide (mg/mL); R-sqr=.80585					
Factor	Regressn Coeff.	Std.Err.	t(3)	p	-90.% Cnf.Limt	+90.% Cnf.Limt
Mean/Interc.	223.11	108.08	2.06	0.1309	-31.24	477.46
(1)Time	240.40	142.98	1.68	0.1913	-96.07	576.88
(2)Temperature	372.42	142.98	2.60	0.0800	35.95	708.89
1 by 2	240.94	142.98	1.69	0.1905	-95.53	577.42

c)

	P07; Regr. Coef.; Var.:Caseinmacropeptide (mg/mL) 2**(2-0) design; MS Residual=2.751233; Adj:.99999 DV: Caseinmacropeptide (mg/mL); R-sqr=1					
Factor	Regressn Coeff.	Std.Err.	t(2)	p	-95.% Cnf.Limt	+95.% Cnf.Limt
Mean/Interc.	385.23	0.83	464.50	0.00000	381.66	388.80
Curvatr.	-378.27	1.27	-298.60	0.00001	-383.72	-372.82
(1)Time	240.40	0.83	289.87	0.00001	236.84	243.97
(2)Temperature	372.42	0.83	449.05	0.00000	368.85	375.99
1 by 2	240.94	0.83	290.53	0.00001	237.38	244.51

Figure 9. *Aeromonas hydrophila* P07 regression coefficients for the dependent variable CMP, showing a representative result of: a) no significant factors in 95% of confidence limit; b) one significant factor in 90% of confidence limit (no model); c) five significant factors after curvature check.
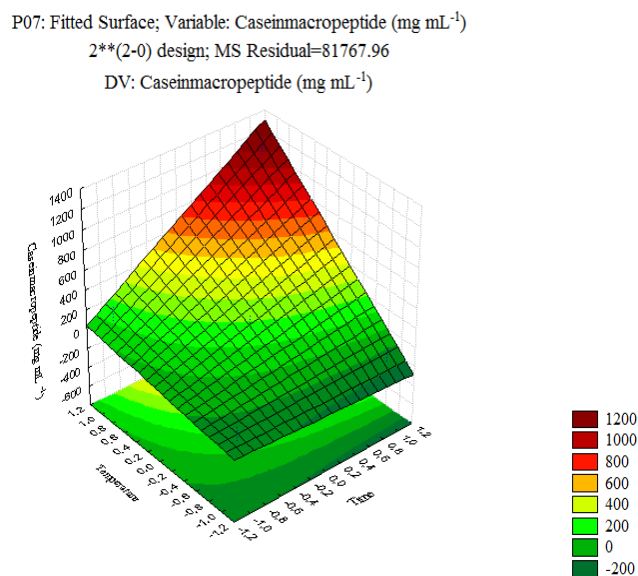
Figure 10. *Aeromonas hydrophila* P07 curve for the dependent variable caseinmacropeptide, representing a model with quadratic tendency.

Table 9. Linear models for the dependent variable caseinmacropeptide (CMP) production by psychrotrophic bacteria that were significant at 90% confidence interval.

Strain	Model	R-sqr	C. Limit	Fcal	Fcrit
P65	243.35 x t + 245.93 x T + 245.50 x t x T	0.874	90%	13.88	4.32
P165	20.51 + 2.92 x T	0.799	90%	4.93	4.06
P272	142.18 + 189.96 x t + 202.44 x T + 192.80 x t x T	0.907	90%	9.79	5.39
P273	145.72 + 209.13 x t + 216.41 x T+ 209.70 x t x T	0.882	90%	7.45	5.39

t = time; T = temperature; C. Limit = confidence limit; Fcal = calculated F value; Fcrit = critical F value.

3.3 Immunoassay Test

The results for selected samples tested by HPLC-GF as well as the results for the same set of samples when analyzed with the immunochromatographic lateral-flow test strip (LFA) are presented in Table 10. A representative image is shown in Figure 11, where the presence of CMP is indicated by a red line, and the intensity of the red color indicates the CMP concentration (30 or 75 mg L^{-1}).

Table 10. Comparison between CMP results by HPLC-GF and immunochromatographic lateral-flow test strip (C-Stick GMP) for a subset of samples (time= 2d).

| Strain	Temperature (°C)	CMP (mg L^{-1})	
		HPLC-GF	Immuno test
Aeromonas hydrophila P07	4	13.35	0
	10	276.30	75
Aeromonas hydrophila P10	4	18.69	0
	10	277.16	75
Aeromonas punctata P65	4	10.30	0
	10	11.17	30
Acinetobacter guillouiae P162	4	10.06	0
	10	12.22	0
Pseudomonas rhodesiae P88	4	10.28	0
	10	26.95	30
Pseudomonas rhodesiae P250	4	10.87	0
	10	369.86	75
Pseudomonas psychrophila P270	4	11.32	0
	10	134.68	75
Pseudomonas psychrophila P272	4	13.28	0
	10	32.55	30
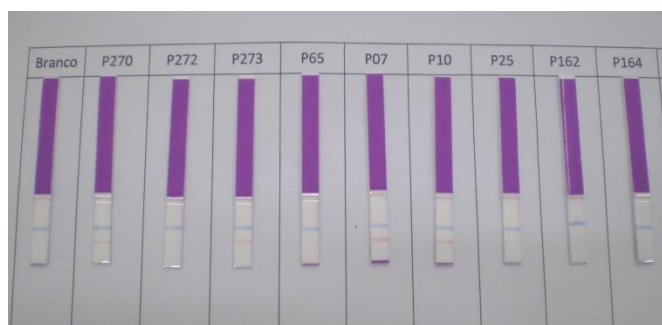
Figure 11. Stick c-GMP (Cap Lab, São Paulo, Brazil) from a previous screening test with milk inoculated with different strains of psychrotrophic bacteria. The red line indicates CMP presence and its intensity indicates the CMP concentration. Branco= blank.

With the exception of *A. punctata* (P65) at the condition 10°C/2d, LFA recognized values lower than 18.69 mg mL^{-1} in the HPLC-GF as a 0 mg mL^{-1} and from this value up to 32.55 mg mL^{-1} as 30 mg mL^{-1}. Values higher than 134.68 mg mL^{-1} were recorded as 75 mg mL^{-1}. Considering that, according to the legislation, values between 30 mg and 74.9 mg mL^{-1} lead to partial condemnation of milk, and ≥ 75 mg mL^{-1} lead to total condemnation (BRASIL, 2006), all of the samples tested (except strain P65) incubated at 10°C/2d would have at least a partial condemnation. In contrast, at the condition 4°C/2d, all milk samples presented CMP

values, as assessed by HPLC and by LFA test, which could allow their direct consumption i.e. (< 30 mg mL^{-1}).

3.4 HPLC-ESI-MS-MS

Our results showed that samples incubated at the condition 10°C/4d presented the highest level of CMP. However, the limitations of this method are well known (MOLLÉ; LÉONIL, 2005; NEELIMA et al., 2013). Hence, for this reason, this subset of HPLC-GF positive samples was selected for HPLC-ESI-MS/MS analysis.

From six monitored ions (Table 11) based on the intensity or efficiency of ionization, calibration curves of two of them (named quantifiers; MAIPPKK 1 and AIPPK 1) were performed after collected them from the HPLC system. These curves had good linear correlation ($R^2 > 0.98$) (Figure 12). However, when the equation was applied in order to know the calculated concentration of those peptides in the samples, peculiar results were obtained such as very low concentrations of CMP and even negative values for pseudo CMP (Figure 13) were achieved. As such, no clear correlation with the HPLC-GF results could be performed. Possible loss of material during the process of collection/extraction of the pure peak may offer a reasonable explanation for this phenomenon.

Table 11. Monitored ions from trypsin digestion of CMP and pseudo CMP.

| Analyte	Ion	Precursor ion	Product ion	Name
MAIPPKK	$[M+2H]^{2+}$	392,8	175,1	MAIPPKK 1
			226,2	MAIPPKK 2
			235,3	MAIPPKK 3
AIPPK	$[M+2H]^{2+}$	263,3	157,1	AIPPK 1
			185,1	AIPPK 2
			341,0	AIPPK 3
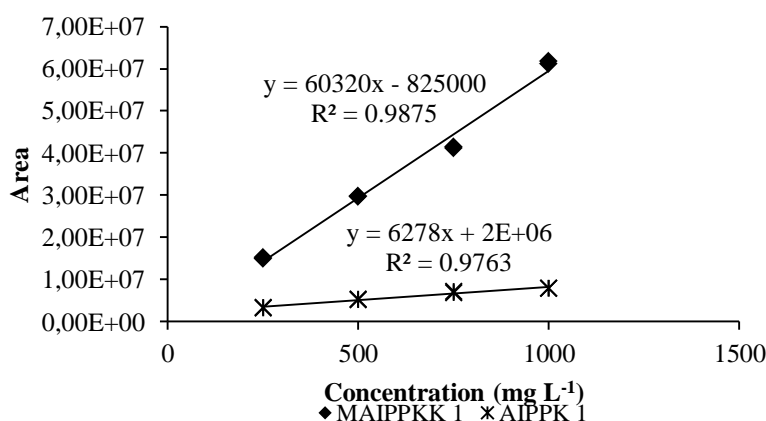
Figure 12. Calibration curves of natural peptides MAIPPKK 1 (CMP) and AIPPK 1 (pseudo CMP) collected from HPLC system and extracted by SPE.
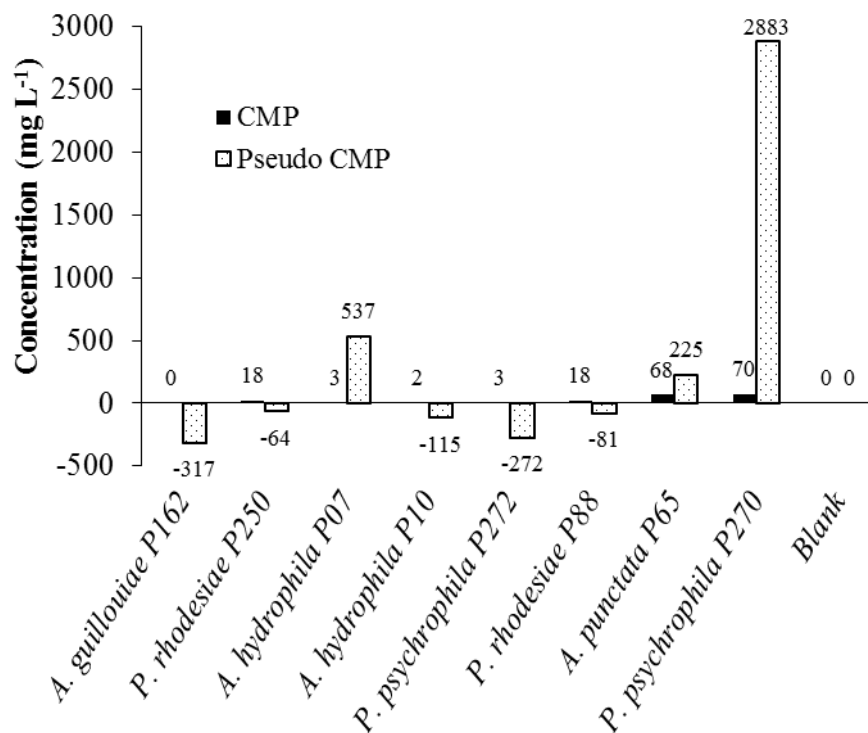
Figure 13. Calculated concentration of CMP and pseudo CMP according to HPLC-ESI-MS/MS analysis by use of linear equation of calibration curve of collected peaks from monitored ions MAIPPKK 1 and AIPPK 1.

Moreover, it is necessary to consider that the lack of a commercial standard for pseudo CMP make it impossible to apply the same treatment to standard and sample, which primarily involves the step of extraction from the matrix (milk), thus allowing a representative comparison. In this context, it was observed that the results of the comparison of CMP concentration by HLPC-GF (which include CMP and pseudo CMP) and MAIPPKK by HPLC-ESI-MS/MS (which include only CMP) using the linear curve of the ion MAIPPKK 1 from the CMP standard in matrix (milk), made sense (data not showed).

Considering the phenomenon explained by the Figure 13, it was decided to present the data as a function of the ratio of the peak areas of the monitored ions. The data from that analysis showed that within the selected subset of samples, only the strain *Aeromonas punctata* P65 presented more than 50% (53.98%) of CMP (and 46.02% of pseudo CMP). The others strains presented a higher percentage of pseudo CMP relative to true CMP (Figure 14). Despite the fact that the storage conditions examined could be considered as extreme, the findings provide a scenario whereby false-positive adulteration with whey could be recorded due to bacterial activity and the producer could receive the improper punishment for the fraudulent practice.
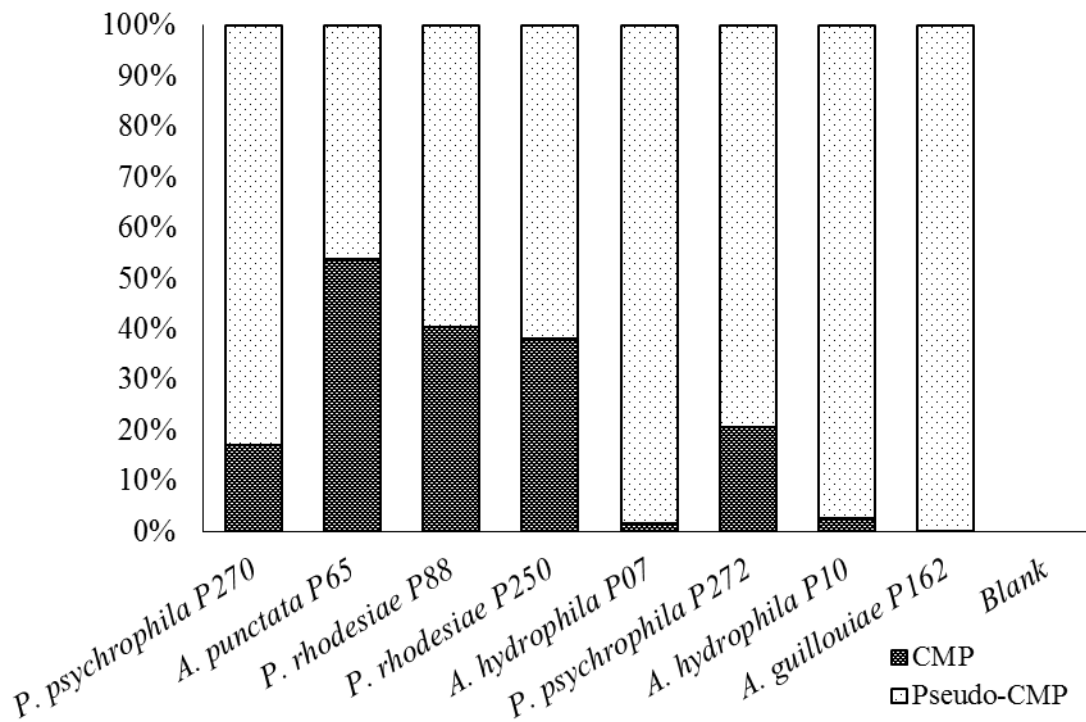
Figure 14. Percentage of CMP and pseudo CMP in a subset of samples (10°C/4d).

3.5 Quantification of Extracellular Proteolytic Activity

The highest proteolytic activity was shown for strains incubated at 10°C/4d, which was correlated with the highest level of growth. In relation to the second highest populations (generally the condition 10°C/2d), the proteolytic attained low levels even when the difference in population number was only lower as one log cycle e. g. (P65). In spite of this limited activity, the tests were performed until the fourth highest population (from seven in total). The results of the first and second highest populations are presented in Figure 15.
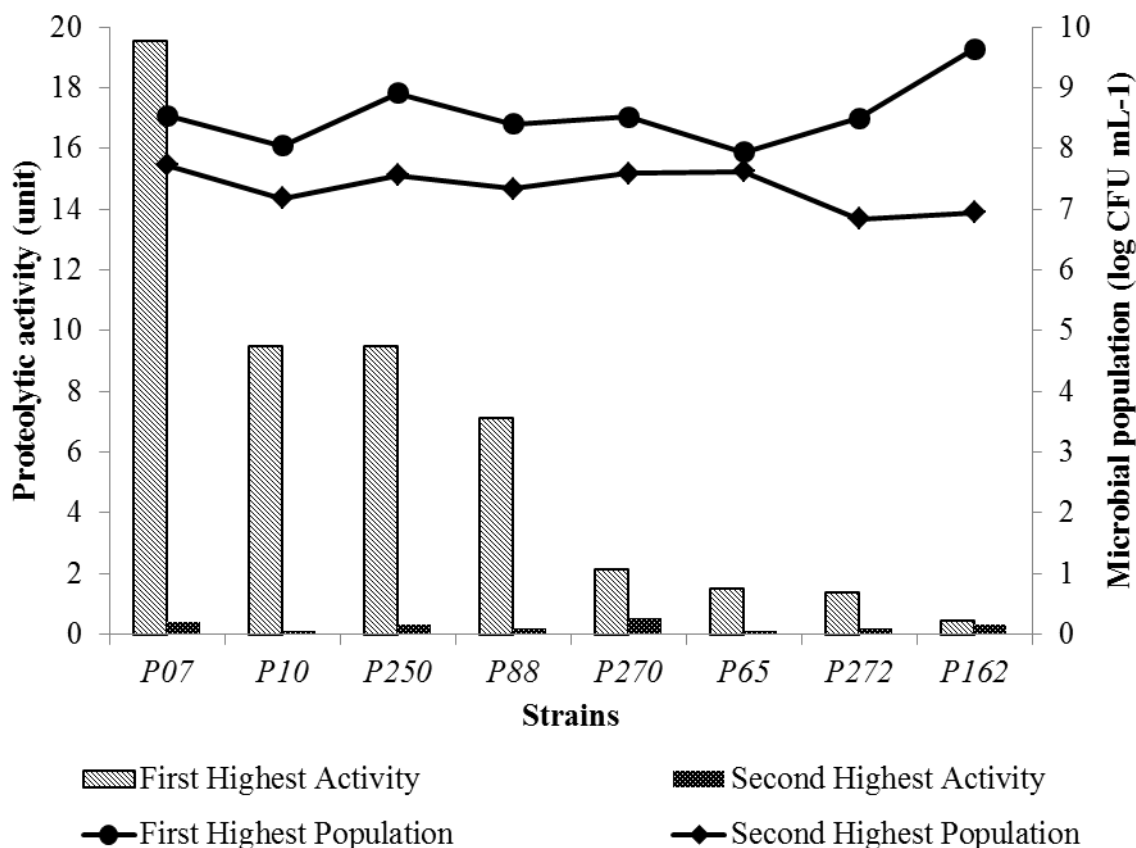
Figure 15. Proteolytic activity for the first and second highest population from the experimental design. 1 unit= amount of enzyme required to produce an increase in absorbance at 420 nm of 0.01 per hour of reaction.

A. hydrophila P07 was shown to exhibit significant proteolytic activity in this test (*ca* 20 units and *ca* 50% higher than the *A. hydrophila* P10 and *P. rhodesiae* P250, which presented similar levels both in the second position). Interestingly, this result was different from that observed in milk agar with respect to CMP levels, where Pseudomonads presented the highest activities.

3.6 Challenge Testing at 30°C

Except for the *A. johnsonii* strain (P25), all samples presented alterations including presence of sediment or apparent proteolysis when incubated under the conditions of 30°C/120d. These effects were most notable in the case of samples incubated at condition 10°C/4d followed by 10°C/2d, 4°C/4d, 7°C/3d and 4°C/2d (Figure 16).
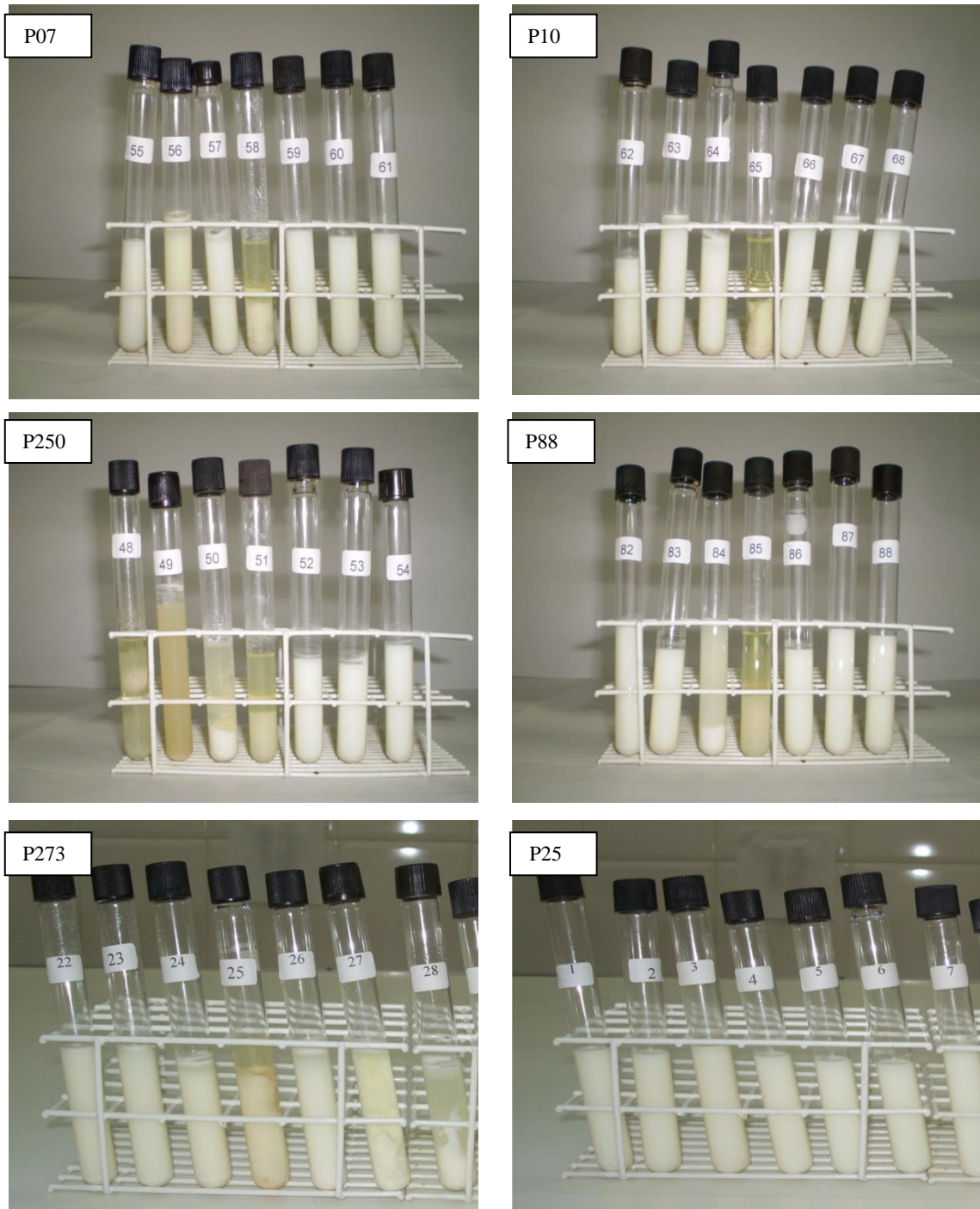
**Figure 16.** Milk samples previously inoculated with *Aeromonas hydrophila* P07, *Aeromonas hydrophila* P10, *Pseudomonas rhodesiae* P250, *Pseudomonas rhodesiae* P88, *Pseudomonas psychrophila* P273, *Acinetobacter johnsonii* P25, and incubated (from left to the right) in each figure in the following order: 4ºC/2d; 4ºC/4d; 10ºC/2d; 10ºC/4d; 7ºC/3d; 7ºC/3d; 7ºC/3d, heat treated (121ºC/3min) and again incubated (30ºC/120d).

Once again, it was possible to confirm that the *Acinetobacter* strains (for example, *Acinetobacter johnsonii* P25) produced no deleterious effect upon the milk, even after two extreme consecutive incubation periods (10ºC/4d within the presence of viable cells and then 30°C/120d with non-viable cells).

4 DISCUSSION

The experiments conducted in this study sought to characterize the growth abilities under refrigeration temperatures of a variety psychrotrophic bacteria isolated from raw milk, and to employ different methods to analyze the production of CMP via the action of the bacterial proteases, in order to extend the range of possible CMP producers. The main objective was to extend our current knowledge in relation to the well-recognized phenomenon of false-positive results, owing to the action of bacterial proteases action, for the practice of fraudulent addition of whey in milk, based on tests which assess the CMP index. It is important to note that, until now, this feature has been studied exclusively using psychrotrophic isolates of *P. fluorescens* (RECIO et al., 1996; RECIO et al., 2000a; MOTTA; HOFF, 2009; OLIVEIRA et al., 2009).

All of the strains demonstrated the lowest populations when exposed to the most restrictive incubation conditions (4°/2d). In this context, a discussion of the importance of the maintenance of milk at lower temperatures during storage and transportation is a highly relevant topic in Brazil. According to Santos and Laranja da Fonseca (2001), unreliable electricity supply conditions in large parts of Brazilian rural properties coupled with the lack of standardization of different milk cooling tank brands resulted in the occurrence of a phenomenon termed "marginal cooling of milk", which signified that milk cooling and storage could take place at temperatures between 5 and 10°C.

Brazilian legislation allows the storage of raw milk up to maximum of 7°C on the dairy farm and at up to 10°C at the dairy processor (BRASIL, 2011). Thus, values considered as a "marginal cooling of milk" are, therefore, legally protected under some situations in a country with tropical temperatures during almost the entire year such as Brazil.

Temperature (and not time) was the most important factor influencing the microbial population growth in our study. This finding corroborates those of Cousin (1982) who stated that temperature is one of the most important variables affecting microbial growth within natural or controlled laboratory environments. In addition, Vyletelova; Hanus and Urbanova (1999) asserted that any handling or storage of raw milk, during which the recommended temperature is exceeded resulted in an increase in the levels of psychrotrophic microorganism, which could attain or surpass the levels recorded for the mesophilic microorganism population. Under natural conditions, the combination of low temperature with low levels of initial contamination makes the population control more efficient (GRIFFITHS; PHILLIPS; MUIR, 1987; CHAMPAGNE et al., 1994).

Whether one can correlate (or should) the characteristics of pure and a mixed bacterial population growth is a controversial issue (PHILLIPS; GRIFFITHS, 1987). As with numerous other studies, our research was conducted from the perspective of pure cultures. Starting from a reasonable population of 3 log CFU mL^{-1}, at the condition 7°C/3d, all strains reached populations higher than 6 log CFU mL^{-1}, except the strain P07. Many studies have commented that this limit, if exceeded, can cause perceptible damage to dairy products (DE NONI; RESMINI, 2005). Importantly, it also confirms the unsuitability of the Brazilian legislation.

In an earlier study, Fajardo-Lira et al. (2000) inoculated two strains of P*seudomonas* sp. (SRM28A and M3/6) into fresh milk. It was observed that starting from the level of 3 log CFU mL^{-1}, the bacterial counts reached $\sim$ 8 log CFU mL^{-1} after incubation at 7 °C for 3 d.

According to Kumaresan; Annalvilli and Sivakumar (2007), reducing the milk storage temperature from 7°C to 2°C significantly reduced the growth of psychrotrophs and decreased both proteolytic and lipolytic activities, providing better sensory qualities when compared to milk incubated at 4°C and 7°C for periods of up to 14 days. Furthermore, Griffiths; Phillips and Muir (1987) stated that storage of raw milk at 2°C resulted in a 1.8 fold increase in storage life compared with that at 6°C. The use of even more restrictive conditions was discussed by Champagne et al. (1994), where the milk with good initial microbial quality (less than 4 log CFU mL^{-1}) kept during 3 d at 1°C, maintained the total count under 5 log CFU mL^{-1}. Despite the obvious advantages, the use of extremely low temperatures is recognized as having intensive energy demands and requires large capital outlay for the equipment employed at each stage of collection, transportation and processing. Curiously, in our challenge test, *P. rhodesiae* P250 in the condition of the sample incubated primarily at 4°C/2d demonstrated more apparently proteolysis than noted in the samples incubated at 7°C/3d. This result matched the data derived in relation to CMP level as determined by GF- HPLC.

Cousin (1982) affirmed that at low temperatures, the growth curve was characterized by an extended lag phase and slower logarithmic phase. This author related the generation times (GT) for a variety of strains of psychrotrophic and psychrophilic bacteria. For example, two strains of *Pseudomonas* sp. required 26.6 and 29.1 h (or 1596 and 1746 min) at 0-2°C, while a strain of *Enterobacter aerogenes* required 37.7 (2262 min). The GT decreased, respectively, to 11.7, 14.7 and 12.2 h at 4-6°C and to 5.43, 6.52 and 4.1 h at 10°C. Our experimental design also focused upon the use of different times, which could include different phases of the curve of microbial development. Owing to this factor, it is difficult for us to make direct comparisons with previous studies, although the temperature ranges are quite similar. In general, our results showed that the condition 4°C/2d presented the highest GT, but at the same temperature during 4 d the GT decreased substantially. Using *P. rhodesiae* (P88 and P250) as examples, it was observed that they had GT's of 15 and 25 h (or 901 and 1522 min) at the condition 4°C/2d, but these values had reduced by around 35% (592 min) and 42% (643 min) at the condition 4°C/4d.

The high levels of CMP were recorded at the condition 10°C/4d, where proteolytic effects highly deleterious to the milk were observed. The two strains of *P. rhodesiae* (P250 and P88) demonstrated the highest levels CMP production (1342 mg L^{-1} and 1289 mg L^{-1} respectively), followed by *A. hydrophila* (P07) with 1239 mg L^{-1}. At the same time, *Acinetobacter* ssp. (P162, P164, P165, P25) did not demonstrate significant increases in the CMP levels despite showing the highest populations (between 7.98 and 9.64 log CFU mL^{-1}). These findings are in agreement with the negative results for proteolysis by *Acinetobacter* sp. in milk agar, confirming the low spoilage potential of this genus and discounting them as possible causes of false-positive whey adulteration results.

The association of *Acinetobacter* sp. and *Pseudomonas* sp. strains predominating in raw milk microbiota has been reported (WIEDMANN et al., 2000; FRICKER et al., 2011). The dynamics of the microbial population in raw and treated milk during cold storage was studied by Rasolofo et al. (2010). According to those authors, in raw and thermized milk (TH) or milk supplemented with CO$_2$ (CO$_2$), *Acinetobacter* was one of the dominant genera in samples incubated from 4 to 8°C for 3 days while the genus *Pseudomonas* was predominant in microfiltrated (MF) milk. However, at the day 7, *Pseudomonas* had become dominant in raw, CO$_2$ and MF milk, while *Staphylococcus sp.* and *Delftia* sp. were dominant in TH milk.

When incubated at the condition 10°C/2d, deleterious effects owing to increased CMP levels were also confirmed in the samples incubated with strains of *A. hydrophila* (P07 and

P10), *P. rhodesiae* (P250) and *P. psychrophila* (P270, P272 and P273). Indeed, the levels of CMP recorded could lead to the milk been classified as fit for partial utilization (if between 30 and 75 mg L^{-1}) or discount the use of the milk as food (>75 mg L^{-1}), according to the Brazilian legislation (BRASIL, 2006). Under this condition, P250 also demonstrated the highest CMP production followed by P07 and P10.

The difficulties found in building linear models for CMP production indicated that no function could be identified which could be a good fit to these data. Possible degradation of CMP molecule, probably as a consequence of activity of the same psychrotrophic bacterial proteases, could be the reason. A similar observation was made by Oliveira et al. (2009) and Recio et al. (2000b).

Although the milk storage at 10°C in the Brazilians dairy farms is not legally permitted, the data from this study indicate that, in addition to causing proteolysis and creating quality control problems in milk and dairy products, psychrotrophic bacteria other than pseudomonads (e.g. *Aeromonas hydrophila* and *Aeromonas punctata*), can also produce CMP and, as a consequence, could theoretically promote interference in some tests used to detect milk fraud owing to whey addition.

Strains of *A. hydrophila* are found regularly in milk and dairy products. According to Santos et al. (1996), who tested the activity of extracellular proteases of two strains of *A. hydrophila* isolated from milk, the presence and importance of *A. hydrophila* in milk and dairy products should not be underestimated. In support of this assertion, the authors reported that for one of the strains evaluated, the addition of milk to the culture medium acted as stimulant for the production of extracellular proteases associated with undesirable effects in milk. *A. hydrophila* is a microorganism widely distributed in fresh and brackish waters, it is a primary pathogen of fish and other aquatic animals and it has been isolated from numerous food products, including raw milk and dairy products. In addition, acting as a spoilage organism, it is also considered as an emerging food-borne pathogen owing to its recognized ability to cause gastro-intestinal infections in humans (DASKALOV, 2006).

In a study performed with 142 isolates of psychrotrophic bacteria isolated from raw milk, one was identified as *Pseudomonas* spp. and nine as *P. aeruginosa* (NÖRNBERG et al., 2010). According to the authors, the *Pseudomonas* isolates did not present relevant levels of proteolytic activity when compared with two isolated strains of *Burkholderia cepacia*, two of *Klebsiella oxytoca* and one of *Aeromonas* sp., which presented expressive and different proteolytic activity also between strains. Isolates of the genera *Serratia* and *Stenotrophomonas* were also considered as a high protease producers among microorganisms isolated from a sanitized dairy plant in study performed by Cleto et al. (2012).

As stated above, the genus *Pseudomonas* has been exalted in many studies, with *P. fluorescens*, *P. fragi* and *P. putida* having been reported as the most abundant species in the microbiota of raw and processed milk microbiota (WIEDMANN et al., 2000). Among the *Pseudomonas* species evaluated in this work, the strains genotypically identified as *P. rhodesiae* (COROLER et al., 1996) were the most important CMP producer (*ca* 1300 mg L^{-1}) according to HPLC-GF results. Yet, at the same time, the three *P. psychrophila* isolates (YUMOTO et al., 2001) demonstrated the capacity to generate CMP, producing *ca* 800 mg L^{-1}. Interestingly, neither of those species has been identified previously as a milk spoilage organism, although *P. psychrophila* was initial identified in milk. The development of new identification techniques based on analysis of molecular markers (discussed in the chapter II) has helped to elucidate some of the complex problems related to the diversity and taxonomy of this genus (PEIX; RAMÍREZ-BAHENA; VELÁZQUEZ, 2009). It should be noted that

ours is not the first study to question the validity of phenotypic based identification of pseudomonads associated with milk spoilage. Other studies have identified species such as *P. fragi* and *P. lundensis* as numerically predominant (53% of the isolates), in raw milk and also demonstrated the ability of these species to produce heat-resistant proteases (MARCHAND et al., 2009b). We agree with the statement made by those authors that the continued application of molecular based identification methods and a subsequent revision of the taxonomy of the *P. fluorescens* lineage will probably result in a different perspective on the role of this organism in relation to the milk spoilage (MARCHAND et al., 2009a).

Although commonly reported as a component of the raw milk microbiota (GRIFFITHS; PHILLIPS; MUIR, 1987; ARCURI et al., 2008; RASOLOFO et al., 2010; FRICKER et al., 2011; VAZ-MOREIRA et al., 2011), some authors also have commented that *Acinetobacter* sp. have a limited spoilage potential, although they are often reported as lipolytic (FITZ-GERALD; DEETH, 1983; VARNAM; SUTHERLAND, 2001).

Some *Acinetobacter* spp. strains, probably belonging to a specific cluster, are also responsible for the occurrence of ropy milk due to the production of a capsular polysaccharide which increases the viscosity of milk and serum (MORTON; BARRETT, 1982; GENNARI et al., 1992; SUZUKI et al., 2001; VARNAM; SUTHERLAND, 2001).

In contrast to our findings with the *Pseudomonas* isolates, our data showed that the four *Acinetobacter* sp. studied were not relevant as either protease producers or in the production of CMP. This result was somewhat unexpected, given the previous characterization of these isolates a proteolytic in milk agar (ARCURI et al., 2008), a feature which lead to our inclusion of these strains in the present work. An additional motivation for including *Acinetobacter* spp., was the study of Hantsis-Zacharov and Halpern (2007) where 29 strains of *Acinetobacter* sp. were recovered from raw milk, and, were shown to have extensive enzymatic activities. The majority of the strains were highly lipolytic, but a number of isolates exhibited both proteolytic and lipolytic activities and a limited number demonstrated exclusively proteolytic activity.

The results discussed above have revealed the possible existence of some previously unidentified players (bacteria), which could interfere in the detection of fraud, when the detection is based upon the CMP index. However, before reaching a definitive conclusion it is of value to point out that the CMP analysis has some particularities. To start with, CMP is a heterogeneous molecule with various forms which differ in terms of their glycosylation level and/or genetic polymorphism (YVON et al., 1994). The reason for the marked heterogeneity of CMP is because it contains all the post-translational modifications (glycosylation and phosphorylation) present in κ-casein (MARTINEZ; FARÍAS; PILOSOF, 2011). Moreover, the concentration of CMP can naturally increase during the storage time in raw and processed milk (e.g. UHT) (CLOSS; SOUZA, 2011).

Several methods have been developed for CMP detection in dairy products including colorimetric, chromatographic, immunological, biosensors, capillary zone electrophoresis (CZE or CE), high performance liquid chromatography – electrospray tandem mass spectrometry (HPLC-ESI-MS/MS), fluorometric and SDS-PAGE (BREMER et al., 2008; CHÁVEZ et al., 2012; NEELIMA et al., 2013). However, not all of these methods have been validated or are currently in use (NEELIMA et al., 2013).

Recently, Fourier transform infrared spectroscopy (FT-IR) (CASSOLI et al., 2011) and lab-on-a-chip electrophoresis technologies (FURTADO et al., 2011) were tested as a non-GMP-based methods, but although they provide several advantages over the traditional

techniques, they still do not present a solution for the detection of fraudulent addition of whey to milk, unfortunately.

Mollé and Léonil (2005) pointed out that the CMP analysis in raw milk has several limitations. The first of these, which was corroborated the observations of Lieske and Konrad (1996) and Thomä; Krause and Kulozik (2006), is the difference in sensitivity to trichloroacetic acid (TCA) precipitation noted for the glycosylated and non-glycosylated forms of CMP, which should be considered when estimating the final CMP concentration in the sample. The second problem is the poor chromatographic resolution based on chromatographic retention time. This problem was also commented upon by Neelima et al. (2013), who affirmed that the heterogeneity of GMP owing to glycosylation creates a problem for the use of methods like RP-HPLC, which do not separate these forms as a single peak. The third difficulty is related to the high dependence of pH for the CZE method. The pH is responsible for self-aggregation due to hydrophobic interactions, which can also be a problem in other methods including as Western blotting (NEELIMA et al., 2013). The last and possibly most important limitation is the occurrence of false-positive results due to the levels of casein denaturation (owing to the action of bacterial enzymes) in dairy products. Indeed, the chances of detecting false-positive results are increased when using the highly sensitive methods (NEELIMA et al., 2013).

Officially, the European Union (EC Regulation, 2008) adopts gel-filtration HPLC (GF-HLPC) and/or reverse-phase HPLC (RP-HPLC) as the method of choice to investigate the presence of CMP in skimmed milk powder. In Brazil, HPLC-GF was the only official method until 2010, at which time both CZE and HPLC-ESI-MS/MS were also adopted by the Brazilian government (BRASIL, 2010). However, despite being officially implanted, both tests are still in the implementation phase.

The use of mass spectrometry (MS) in the analysis of food products has allowed the accurate determination of molecular mass and protein sequences, the detection of post-translational modifications (phosphorylation and glycosylation) or chemical modifications of primary sequences, the detection of new genetic variants, identification of protein degradation products, and the study of protein conformations (DEL MAR CONTRERAS et al., 2008).

As reported by various authors, HPLC-ESI-MS/MS (and also CE) are the only methods capable of differentiating between genuine fraud and bacterial degradative action on milk samples, with MS providing the ultimate specificity (VAN RIEL; OLIEMAN, 1995; RECIO et al., 2000a; RECIO et al., 2000b; DE NONI; RESMINI, 2005; MOLLÉ; LÉONIL, 2005; BREMER et al., 2008). The application of these methods allows CMP to be distinguished from pseudo CMP, a molecule which is generated via the cleavage of the bond 106-107 and has only one methionine less than CMP (DE LA FUENTE; JUÁREZ, 2005; BRASIL, 2010). It is important to note that the presence of pseudo CMP in conjunction with CMP, which may also be produced by bacteria, is considered to be indicative of fraud, although, owing to secondary reactions of chymosin (HOME; BANKS, 2004), careful quantitative analysis is required.

According to De La Fuente and Juárez (2005) and Van Riel and Olieman (1995), CE has two main advantages: a) very high resolution of proteins and peptides that differ by just a single amino acid residue with no limitation in relation to the size of the components to be separated, unlike conventional electrophoresis; b) potentially, it provides rapid separation because the adsorption from the proteins to the capillary wall is suppressed.

CE can be useful for both discriminant and quantitative analysis, but, in the Brazilian legislation (BRASIL, 2010) this step can be suppressed and the samples confirmed positive in the screening assays (HPLC-GF) can be directly analyzed by HPLC-ESI-MS/MS.

In electrospray ionization coupled to HLPC-MS/MS, as required for the Brazilian method, the analytes are ionized at atmospheric pressure directly from a flowing liquid stream, and the ions produced are then directed into the mass spectrometer. Liquid from either an infusion pump or HLPC effluent enters into the atmospheric pressure ionization source through a capillary restriction held to high voltage (±3-5 kVs). The high electric field at the tip of the needle causes the solution to disintegrate into an aerosol plume of very small electrically charged droplets, in a process referred to as electrospray. The protein can gain a lot of charges due to the presence of charged amino acid residues and the molecular mass of the protein may be calculated by deriving the charge states of any two adjacent ions in the series (LÉONIL et al., 2000).

The sample preparation in the Brazilian methods involves previous enzymatic digestion of CMP by trypsin (Figure 17) which converts CMP and, if present, pseudo CMP, into a peptide with 6 or 7 amino acids (initial portion), eliminating the glycosylation, phosphorylation and genetic mutation issues associated with the CMP molecule (MOTTA; HOFF, 2009).
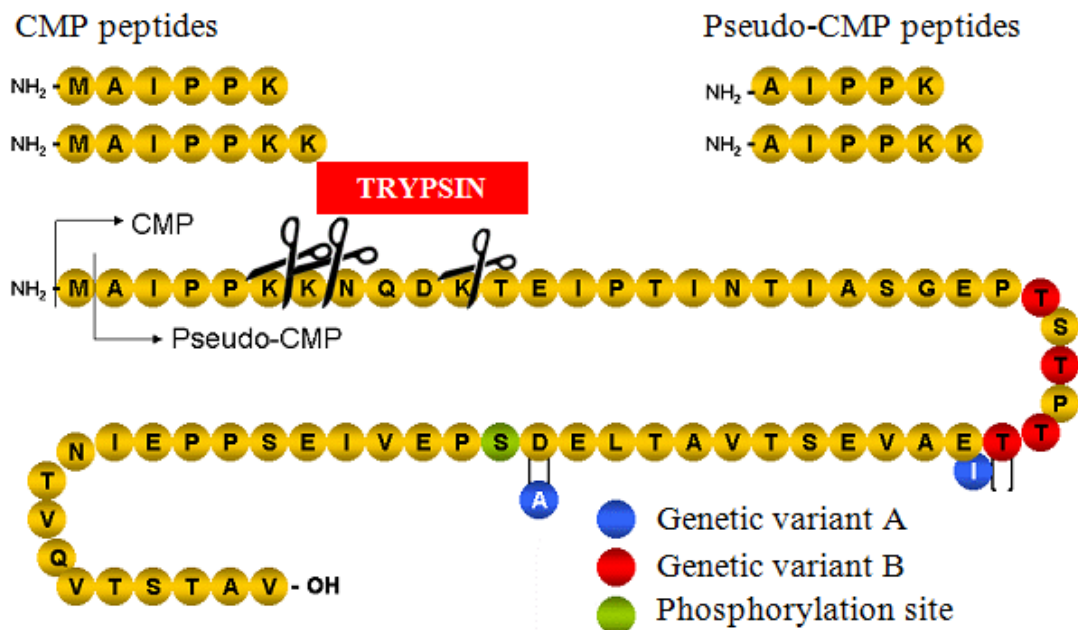

Figure 17. Cleavage sites of enzymatic digestion of CMP and the pseudo CMP by trypsin. Four small peptides are generated: from partial cleavage - MAIPPKK (CMP) and AIPPKK (pseudo CMP); and from full cleavage - MAIPPK (CMP) and AIPPK (pseudo CMP). Source: MOTTA (2013).

A similar digestion strategy was also used by Mollé and Léonil (2005) with the same objective i.e. identify a fragment common to all CMP forms (genetic variants A and B) and end-product with a 100% splitting site yield. The objective of those authors was quantification

of total CMP$_{AB}$. However, rather than trypsin they used bovine pepsin, which released the C-terminal κ-casein (162-169) peptide.

The results achieved by use of HPLC-ESI-MS/MS in our samples showed that the pseudo CMP molecule (or peak) collected from the HPLC system did not provided reliable results, hampering the analysis of the concentration in mg L^{-1}. This problem could be partially resolved with the infusion of a standard the pseudo CMP peptide, which is already foreseen for the next steps of implementation of this technique at the Laboratório Nacional Agropecuário (LANAGRO). In the absence of such standard, and as an alternative option, we decided to present the data according to the peak area ratio, which is a plausible solution, considering that areas are also quantitative. In this way, we could observe that large amount (>50%) of CMP which were detected by HPLC-GF, were, in fact, pseudo CMP, with this result being less marked only in the case of *A. punctata* (P65).

Also using the peak area ratio of different CMP-like components, Recio et al. (2000b), aiming to check if using these ratios could be applied to the detection of rennet whey solids in UHT milk, incubated raw milk at 4°C/24h (according to them, sufficient time for the growth of psychrotrophic bacteria and the production of thermostable proteases) and then adulterated this milk with reconstituted whey powder at a final concentration of 6.4%. The mixture was heat treated (137°C/4s), incubated at 10, 20 and 30 ± 2°C, and analyzed by EC at zero, 15, 30, 60, 90 and 120 d. According to those authors, EC allows the separation of the three most important forms of CMP and the results were: a) on day zero only one peak of CMP (CMP2= true CMP) was detected; b) at 10°C the CMP2 remained constant and only after 30 d appeared a peak of CMP3; c) at 20°C CMP3 peak was present at 15 d and after 60 d there was a parallel increasing of CMP1 and CMP3 while CMP2 started to decline after 90 d; d) at 30°C CMP2 had a rapid decline followed by the appearance of CMP3 and elevation CMP1. After 90d, the electrophoresis showed high proteolytic degradation, which interfered with the accuracy of the integration of the peak. They concluded that using CE peak area ratios of these three peptides could allow the detection of rennet whey solids added to milk, except in the case of extensive proteolysis caused degradation of the CMP2, which can modify the normal CMP-like peak ratios.

Recio et al. (2000a) also performed the separation between CMP derived from chymosin and pseudo CMP derived from the action of extracellular protease of *P. fluorescens* strain (B52). Separation of the molecules was performed initially by HPLC-RP. The milk treated with chymosin showed three peaks with expected retention time for CMP. Two of the peaks represented the non-glycosylated and monophosphorylated forms of both genetic variant (A and B) of CMP. Two major peaks were detected in the milk hydrolyzed with extracellular protease of *P. fluorescens*. Major peaks were collected from HPLC system and then reanalyzed by CE. The identity of the peptides was performed by ESI-MS. According to these authors the peaks originated from the proteolytic activity of *P. fluorescens*, for both κ-casein A and B, were: a) CMP molecule; b) CMP lacking N-terminal Met-106 residue; c) CMP lacking N-terminal Ala-107 residue; d) Phe 105–CMP and e) Ser-104-Phe-105 –CMP. They highlighted that the presence of the two last forms varied depending on the time of hydrolysis and concluded that pseudomonads proteases were less specific than chymosin (which cleaves 105-106 bond of casein) and also cleave bonds 103-104, 104-105,106-107 and 107-108.

The pre-requisites of costly equipment and highly qualified labor and the relatively long time required to obtain results are always negative points of any analysis. To address this issue, the use of rapid screening tests, especially in inspection process, can be considered to

represent an advance. Immunological (and other rapid) tests have been developed for several analytical tests with milk, e.g. for detection of antibiotic residues (TENÓRIO et al., 2009) and alkaline phosphatase detection (VEGA-WARNER et al., 2000). In the specific case of fraud, these tests has been used mainly for the detection of milk mixtures from different species in dairy products (VELOSO et al., 2002) and, more recently, to detect melamine, owing to a milk fraud scandal in China involving the incorporation of melamine in milk powder (GARBER, 2008; YIN et al., 2010). The relative simplicity of the tests and their high sensitivity due to the specificity of the antigen-antibody reactions, associated with the use and low cost and unsophisticated equipment, clearly shows that these methods hold great potential for application in the food industry (VELOSO et al., 2002).

Firstly developed for use in milk and buttermilk powders (BREMER et al., 2008), the test (LFA), as used with our samples had its performance tested by Martín-Hernández et al. (2009) using 60 Brazilian raw milk samples spiked with 0, 1, 2, 4 and 16% v/v of whey. The results were compared with HPLC-GF. The test is based on the binding of casein glycomacropeptide (c-GMP) by two specific anti-bovine k-casein monoclonal antibodies and had (in the test) a visual detection limit of around 15 ng mL^{-1} for c-GMP and 1% (v/v) for rennet whey in milk using standards and spiked samples. According to these authors, the results achieved by LFA were reliable in all raw milk samples with whey content above 4% and, in general, the results were similar to those from HPLC. Two weak positive results were found in samples with 4.5 and 5.6% of whey. These authors also reported some discrepancies in samples with a whey content close to the quantification limit of the HPLC-GF test and the detection limit of the dipstick (in the range between 2 and 2.5%, as determined by HPLC), which could be recorded as a false negative if only LFA was used. These authors also commented that a limitation of LFA was the use of this test in milk with poor bacterial quality.

Our results clearly could show false-positive results for fraudulent addition of whey in milk, the main purpose of the LFA test. This test could still serve as a useful evaluator of milk quality with regards to bacterial proteolysis. According to the manufacturer, only high values may characterize fraud (although the test has maximum limit detection equal to 75 mg mL^{-1}). It is noteworthy that, in Brazil, CMP values between 30 mg and 74.9 mg mL^{-1} only lead to partial condemnation of milk, and it is necessary for level to be $\geq$ 75 mg mL^{-1} to result in total condemnation (BRASIL, 2006). Thus, the test corresponds to the interests of this law, providing a satisfactory indication as to proteolysis status, albeit independent of the CMP origin.

LFA was also used by Oancea (2009) in a single raw milk sample and nine commercial milk/dairy beverages samples (pasteurized milk, condensed milk, powdered milk, and dairy beverages of cappuccino, chocolate, banana and strawberry flavors). According to them, the results obtained showed whey addition (1-2%) in 70% of the samples (raw and pasteurized milk and milk drinks). These authors commented the possibility of false-positive results owing to bacterial proteases and explain that only samples properly kept at low temperature and with excellent microbial quality were considered. However, considering that these enzymes are thermostable, the microbiological quality of processed dairy products is not a good quality indicator for this type of test.

A sandwich ELISA for GMP detection was also developed by Chávez et al. (2012) using a polyclonal rabbit anti-GMP antibody. In this test, the objective was the detection of GMP in raw milk. According to these authors this test had lower a detection limit and quantification limit and was considered useful as a routine test in milk processing industries

and even in official inspection programs. However, problems with false-negative results were not discussed by these authors.

According to Nörnberg; Tondo and Brandelli (2009) the methods available for the determination of proteolytic activity in milk are different and usually do not allow a direct comparison of results. Another possibility to access the proteolytic activity of extracellular proteases produced by bacteria is evaluating it in the presence of a substrate. In general, the tests are performed using an enzyme suspension from a culture medium (without cells) (RAJMOHAN; DODD; WAITES, 2002; DUFOUR et al., 2008). However, the determination of proteolytic activity in milk systems (whole, skimmed and ultrafiltrate) has been validated by Bendicho et al. (2002), who also affirm that azocasein is one of the best substrate to measure proteolytic activity, while fluorescein isothiocyanate-casein (FITC-casein) was considered the best option to detect protease activity in UHT milk for Button et al. (2011). In our study we decided to perform this test in our milk samples starting from the highest to the lowest microbial population. Curiously, in this test, a strain of *A. hydrophila* (P07) presented expressive results (almost 50% more) comparing to the second highest activity, therefore, surpassing *P. fluorescens*, which presented the highest levels of CMP production, besides the higher proteolytic performance (based on halo sizes) on milk agar tests. Disagreement between proteolytic activity estimated by the hydrolysis halo in milk agar plates and the activity measured by the azocasein method was also found by Vazquez et al. (1994).

The milk agar test was also used by Wang and Jayarao (2001) to evaluate 55 strains of *Pseudomonas* spp. isolated from milk and which had been identified by phenotypic and genotypic characteristics. They concluded that 80%, 91% and 58% of the strains were proteolytic, respectively at 7°C, 22°C and 32°C. According to these authors, probably, the incubation temperature at 32°C was beyond the optimum temperature, and the proteolytic activity could be inhibited to a certain degree. Similar results, but using a different method, were presented by Santos et al. (1996), who found proteolytic activity in the extracellular products of *A. hydrophila* strains to be higher at 4°C than at 28°C. Our results from the incubation at 30°C/120d also showed that the condition of the sample incubated primarily at 4°C/2d had more apparently proteolysis than samples incubated at 7°C/3d for the strain *P. rhodesiae* P250, which agreed combining with the results of HPLC-GF and indicated enhanced proteases activity at this temperature even at lower numbers of UFC mL^{-1}.

From 142 psychrotrophic bacteria isolated of raw milk in Southern Brazil by Nörnberg et al. (2010), 25 showed halos above 10 mm in milk agar plates at 37°C and also showed halo at 7°C. The azocasein test was performed, with these 25 strains in casein broth medium, and the majority showed azocaseinolytic activity lower than 10 U mL^{-1}. However, five strains showed expressive values: *Burkholderia cepacia* 1A4 (80±11 U mL^{-1}), *Klebsiella oxytoca* 8B3 (55±20 U mL^{-1}), *Burkholderia cepacia* 2A7 (63±2 U mL^{-1}), *Aeromonas* sp.10B7 (35±12 U mL^{-1}) and *Klebsiella oxytoca* 1A5 (53±2 U mL^{-1}).

Kohlmann et al. (1991) tested extracellular protease activity after growth of six psychrotrophic bacteria strains in milk at 7°C during 13 and 20 days. They found an increase in unit activity from day 13 to 20 as 7 to 26 (*P. putida*), <1 to 12 (*Acinetobacter* sp.), 46 to 83 (*P. fragi*), 13 to 5 (*Alicaligenes faecalis*), 40 (not determined on day 20) (*P. fluorescens*), and <1 to 22 (*Chromobacterium* sp.). It was noted in this study that pseudomonads presented the highest proteolytic activity, mainly represented by *P. fragi* (46 and 33 on day 13 and 20, respectively) and even *Acinetobacter* sp. showed proteolytic activity at the day 20. Curiously, in the study performed by Button et al. (2011), the greatest level of proteolysis in UHT milk

occurred following the addition of crude protease from *P. fluorescens* isolate 117 while no increase in proteolysis was seen in UHT milk containing crude protease from *P. fragi*.

5 CONCLUSION

The possibility of false-positive results in the CMP based methods to detect fraud in milk by whey addition owing to the action of bacterial proteases was, as in previous studies, confirmed in all studied methods. At the same time, the sovereignty of *Pseudomonas* sp. was challenged and, by all indications, *Aeromonas* spp. are also capable of interfering in this test causing false-positive results.

Even though our highest CMP concentrations for all psychrotrophics strains (except acinetobacters) were found in milk held under extreme storage temperature conditions (10°C) during 2 and 4 days, we consider them to be indicative of a possible risk. In a tropical country, with a large territory and still adapting to the new rules for milk cooling, we would urge regulatory authorities to think about how best to develop milk quality improvement programs and to undertake investments in detection methods aimed at effectively combating this fraud which continues to defy authorities and researchers worldwide.

HPLC-ESI-MS/MS is the best method developed so far based on the CMP index for this purpose, and represents the only test capable of providing a fair evaluation and consequently the appropriate penalty to the fraudster, although its use is still not a reality.

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3 CONCLUSÃO GERAL

- Fraude em alimentos é um problema que atinge proporções mundiais e que precisa ser combatido. No Brasil, fraude em leite, inclusive por adição de soro, é um problema recorrente, o que aponta a necessidade de melhorias no sistema de inspeção no que se refere a equipamentos e métodos de detecção, assim como medidas preventivas;

- Métodos genotípicos de identificação microbiana têm proporcionado mudança de paradigma e apresentado novos gêneros e espécies de bactérias presentes na microbiota do leite. Da mesma forma, têm propiciado o conhecimento da diversidade de pseudomonídeos, tido como predominantes. Por exemplo, neste trabalho, a identidade de três linhagens de *P. putida*, definida por métodos fenotípicos, foi contestada pela oriunda da identificação genotípica como sendo *P. psychrophila* que, inclusive, foi reportada pela primeira vez em associação com o leite, assim como a identidade de duas linhagens de *P. fluorescens* foi contestada como sendo *P. rhodesiae*;

- O sequenciamento das linhagens de psicrotróficos estudadas (n = 12) mostrou concordância mínima de gênero com a identificação fenotípica, proporcionou identificação em nível de espécie para quatro linhagens de acinetobacters e rejeitou a identificação em nível de espécie para seis linhagens. Apesar disso, as novas identidades não são consideradas definitivas;

- Cromatografia líquida com filtração em gel (HPLC-GF), assim como o método imunocromatográfico rápido usados nesta pesquisa, detectaram o pseudo CMP, oriundo da ação proteolítica das bactérias estudadas, e provavelmente CMP verdadeiro, considerando que estas proteases também podem exercer ação semelhante ao coalho, englobando estes resultados o que, em uma análise oficial, poderia caracterizar fraude no leite por adição de soro. Apesar disso, tais testes se mostraram adequados como teste de varredura para a pesquisa da presença de CMP devido à fraude ou proteólise bacteriana;

- Dentre os não pseudomonídeos estudados, o potencial proteolítico de *A. guillouiae* e *A. johnsonii* foi muito baixo e considerado insignificante, enquanto proteases produzidas por *A. hydrophila* e *A. punctata* foram capazes de produzir CMP, o que as caracteriza como capazes de degradar o leite e gerar resultados falso-positivos em pesquisa de fraude por adição de soro;

- A produção de CMP, mais expressiva na condição de incubação 10°C/4 dias, não seguiu comportamento linear dentro dos limites de tempo e temperatura estudados para oito linhagens de bactérias estudadas, provavelmente devido à contínua degradação da molécula pelas mesmas proteases que a geraram;

- HPLC-ESI-MS/MS, método capaz de diferenciar alterações diminutas em moléculas proteicas, usado segundo metodologia proposta pela legislação brasileira, que inclui digestão da molécula de CMP e pseudo CMP, apresentou resultados promissores.