SOLIMAR GONÇALVES MACHADO

Serratia AND *Pseudomonas* AS IMPORTANT HEAT-RESISTANT PROTEASE PRODUCERS IN COLD RAW MILK

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Doctor Scientiae*.

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RESUMO

MACHADO, Solimar Gonçalves, D.Sc., Universidade Federal de Viçosa, julho de 2015. ***Serratia* e *Pseudomonas* como importantes produtores de proteases termorresistentes em leite cru refrigerado**. Orientadora: Maria Cristina Dantas Vanetti. Coorientadora: Denise Mara Soares Bazzolli.

A estocagem de leite cru sob temperatura de refrigeração não impede o crescimento de bactérias psicrotróficas produtoras de proteases termorresistentes que comprometem a qualidade e reduzem a vida de prateleira de produtos lácteos. Para minimizar os problemas tecnológicos resultantes da atividade proteolítica, é desejável que proteases sejam detectadas e quantificadas em leite cru por um método rápido. No entanto, não existe um método eficiente para este fim. Este trabalho teve como objetivos identificar as espécies psicrotróficas com potencial de deterioração predominantes em leite cru brasileiro; identificar e caracterizar as proteases resistentes ao calor produzidas por esta microbiota e desenvolver um método rápido para detectar estas proteases em amostras de leite. As condições de armazenamento sob refrigeração foram simuladas e as bactérias psicrotróficas proteolíticas foram isoladas. As bactérias produtoras de proteases termorresistentes foram agrupadas após análise por Rep-PCR. Os testes bioquímicos e o sequenciamento dos genes rDNA 16S e *rpoB* foram utilizados para identificar representantes de cada grupo. Uma protease termorresistente produzida por *Serratia liquefaciens* foi caracterizada e o gene que codifica esta enzima foi identificado após análise dos peptídeos trípticos da protease por espectrometria de massa. Placas de microtitulação revestidas com caseína marcada com biotina foram utilizadas para quantificar a atividade proteolítica de amostras de leite e de proteases diluídas em solução tampão. Os isolados bacterianos altamente proteolíticos foram separados em oito grupos diferentes e quatro padrões únicos. Os isolados com potencial proteolítico são correspondentes à espécie *Serratia liquefaciens* e ao gênero *Pseudomonas*. Isolados de *S. liquefaciens* podem produzir uma metaloprotease termorresistente ao tratamento de 95 °C por 8,45 min, identificada como Ser2, com massa molar de, aproximadamente, 52 kDa e semelhante à protease AprX de *Pseudomonas* spp. Embora sequências de nucleotídeos do gene *ser2* sejam conservadas entre os isolados de *S. liquefaciens*, o potencial de deterioração das estirpes é heterogêneo indicando diferenças nos níveis de expressão gênica, enzima

ou modificações pós-transcricionais. O imunoensaio desenvolvido neste estudo foi eficiente para a quantificação da atividade de tripsina, papaína, pepsina, termolisina e protease de pâncreas bovino. No entanto, novas pesquisas devem ser realizadas para minimizar a influência dos componentes da amostra de leite nesta técnica para permitir a detecção acurada de proteases. Este trabalho destaca o elevado potencial de deterioração da microbiota encontrada em amostras de leite cru, além do desenvolvimento de um ensaio promissor, útil para a indústria de lacticínios, para detecção e quantificação de atividade proteolítica resultante das práticas agrícolas inadequadas nas propriedades produtoras de leite.

ABSTRACT

MACHADO, Solimar Gonçalves, D.Sc., Universidade Federal de Viçosa, july 2015. ***Serratia* and *Pseudomonas* as important heat-resistant protease producers in cold raw milk**. Adviser: Maria Cristina Dantas Vanetti. Co-adviser: Denise Mara Soares Bazzolli.

The storage of raw milk at cold temperatures does not prevent growth of psychrotrophic bacteria, which can produce heat-resistant proteases that compromise the quality and reduce the shelf life of dairy products. To minimize the technological problems resulting from proteolytic activity, these enzymes should be detected and quantified in raw milk before processing by a rapid method. However, there is no efficient method for this purpose. This work aimed to identify the predominant psychrotrophic species with spoilage potential in Brazilian raw milk, to identify and characterize the heat-resistant proteases produced by this microbiota and to developed a rapid method to detect these proteases in raw milk samples. The cold storage conditions were simulated and the psychrotrophic proteolytic bacteria isolated. The heat-resistant protease-producing bacteria were typing by Rep-PCR and clustered. Biochemical tests, 16S rDNA and *rpoB* gene sequencing were used for identifying one representative isolate from each cluster. The heat-resistant protease produced by *Serratia liquefaciens* was characterized. The encoding gene was identified after mass spectrometry analysis of tryptic peptides from the heat-resistant protease. The biotinylated casein was coated on microtiter plates and used as substrate to quantify proteolytic activity in solution and milk samples. Highly proteolytic strains were identified and characterized. The isolates were separated into eight different clusters and four solitary fingerprints. The most proteolytic isolates belonged to *Serratia liquefaciens* and *Pseudomonas* species. The *S. liquefaciens* isolates may produce Ser2, which is a a metalloprotease resistant to the heat treatment of 95 °C for 8.45 min. This metalloprotease showed a molecular weight of approximately, 52 kDa and a heat-resistance similar to AprX from *Pseudomonas* spp. Although nucleotide sequences of *ser2* gene were conserved among *S. liquefaciens* isolates, the spoilage potential among them was heterogeneous indicating differences in enzyme expression levels or post-transcriptional modifications. The developed immunoassay was efficient for quantification of trypsin, papain, pepsin, thermolysin and protease from bovine

pancreas activity. However, further research should be performed to minimize the influence of milk components on the developed assay for detecting proteases in milk samples. This work highlighted the poor conditions of hygiene in milk farms and the high spoilage potential of the microbiota found in raw milk samples besides the development of a promising assay for detection and quantification of proteolytic activity useful for dairy industry.

GENERAL INTRODUCTION

In 2002, the Brazilian government established that raw milk should be stored at low temperatures (between 4 and 7 °C) in the farms in order to minimize the growth of mesophilic microbiota (Brasil, 2002). This practice reduces the rate of spoilage by mesophilic microorganisms, but does not prevent the proliferation of psychrotrophic bacteria that can grow at temperatures between 0 °C and 7 °C (Sørhaug and Stepaniak, 1997). The psychrotrophic counts, which is approximately 10 % of the total count of mesophilic aerobes immediately after milking, may reach, on average, 90 % after cold storage (Catanio et al., 2012; Pinto, 2004; Sørhaug and Stepaniak, 1997), although Malacarne et al. (2015) demonstrated that psychrotrophic bacteria represented 67 % of total bacteria in milk stored for 24 h at 10-13 °C.

Several studies have investigated the bacterial population structure and dynamics of cold raw milk (De Jonghe et al., 2011; Decimo et al., 2014; Raats et al., 2011; Rasolofo et al., 2010). Usually, the dominant microbiota found in raw milk belongs to the gram-negative genera *Pseudomonas*, *Achromobacter*, *Aeromonas*, *Hafnia*, *Enterobacter*, *Serratia*, *Alcaligenes*, *Burkholderia*, *Chromobacterium* e *Flavobacterium* spp and gram-positive *Bacillus*, *Clostridium*, *Corynebacterium*, *Streptococcus*, *Lactobacillus* e *Micrococcus* spp. (Munsch-Alatossava and Alatossava, 2006; Pinto, 2004; Rasolofo et al., 2010; Sørhaug and Stepaniak, 1997; Vithanage et al., 2014).

Raats et al. (2011) showed the bacterial population in raw milk samples after 24 h of cold storage presented a complex profile in which *Firmicutes* was the predominant phylum. However, when raw milk samples were stored at cold temperatures for 48 h, there was a significant change in the bacterial community composition and the phylum *Proteobacteria* became dominant. These authors demonstrated that *Pseudomonas* was the most abundant genera found in cold raw milk samples such as De Jonghe et al. (2011), Dogan and Boor (2003), Ercolini et al. (2009), Rasolofo et al. (2010) and Tremonte et al. (2014).

Most of psychrotrophic bacteria found in raw milk are inactivated by heat treatment commonly used in dairy industries such as pasteurization and ultra-high temperature (UHT). However, these microorganisms may secrete proteolytic enzymes, which can be heat resistant. According to Marchand et al. (2008), 75 % of heat-resistant proteases remain active in milk samples after heat treatment at

95°C for 8.45 min. Zhang et al. (2015) showed more than 39.36 % of protease activity remained in milk samples after treatment at 130 °C for 3 min.

The heat-resistant protease produced by *Pseudomonas* spp. is the main enzyme related to milk spoilage targeted in the literature (Baglinière et al., 2013; Dufour et al., 2008; Liao and McCallus, 1998; Marchand et al., 2009b). This enzyme is a metalloprotease codified by *aprX* gene located at *aprX-lipA* operon, which contains eight genes and spans 14 kb (McCarthy et al., 2004). It is well known that *aprX* is expressed mainly at the end of logarithmic phase and during stationary phase when bacterial counts reach 10^6 CFU mL^{-1} or higher in milk residues (Dufour et al., 2008; Sørhaug and Stepaniak, 1997). Moreover, AprX may hydrolyze the four types of casein (α_{s1}, α_{s2}, β, e κ) with a large activity spectrum (Baglinière et al., 2013).

Although AprX is the most studied protease produced by the microbiota found in raw milk, other species isolated from milk samples may also produce proteases such as *Burkholderia cepacia* (Nörnberg et al., 2011), *Serratia liquefaciens* (Decimo et al., 2014), *Staphylococcus aureus*, *Staphylococcus intermedius*, *Staphylococcus warneri*, *Streptococcus uberis* and *Bacillus coagulans* (Teh et al., 2011). *Bacillus* spp. show more diverse proteolytic activity than *Pseudomonas* spp., and many species may secrete more than one type of extracellular and intracellular protease (Nabrdalik et al., 2010).

The intensity of proteolytic changes is dependent on species and stains. Marchand et al. (2009b) and Baglinière et al. (2012) revealed a large heterogeneity regarding *aprX* gene from *Pseudomonas* genus and destabilization of UHT milk inoculated with *P. fluorescens* strains, respectively. The high variability of *Pseudomonas* strains regarding the proteolytic activity may be a consequence of heterogeneous enzyme expression, regulation by quorum sensing, effect of temperature, iron content and bacterial growth phase (Marchand et al., 2009a; Nicodème et al., 2005; Woods et al., 2001).

The heat-resistant protease can lead to a serious problem for the dairy industry. Pinto et al. (2014) showed that α-, β- and κ-casein from milk inoculated with *P. fluorescens* were completely hydrolyzed after 4 days incubation at 4 °C. Other technological problems in the dairy industry caused by proteolytic activity from psychrotrophic microorganisms have been highlighted such as the gelation of

UHT milk, the thermal instability of milk, the off-flavor of some dairy products and a yield reduction in cheese manufacturing (Beyer et al., 1998; Cardoso, 2006; Celestino et al., 1997; Pinto, 2004; Samaržija, 2012). In view of these problems, it would be desirable to develop a rapid, sensitive and simple method to predict the shelf life of dairy products.

The standard plate count procedure is often employed to detect psychrotrophic contamination in milk and dairy products. However, this method is time-consuming and does not allow for the rapid assessment of food spoilage potential (Martin et al., 2011). An alternative is the detection of the gene encoding the heat-resistant protease. Martins et al. (2005) proposed a method for detection of *aprX* gene using polymerase chain reaction (PCR) aiming the assessment of spoilage potential in milk samples. Even with improvements (Machado et al., 2013; Marchand et al., 2009b), this method based on detection of protease encoding gene is not appropriate to assess spoilage potential since there is an heterogeneity of proteolytic activity of *P. fluorescens* strains having *aprX* gene in the genome (Baglinière et al., 2012; Dufour et al., 2008; Marchand et al., 2009b).

Immunology has also been used as a tool to develop methods for dairy industries (Dupont et al., 2007; Machado, 2006; Matta et al., 1997). However, since heat-resistant proteases are heterogeneous, the production of one antibody for detection of proteases produced by the dominant psychrotrophic proteolytic bacteria is challenging.

Considering there are other important groups of psychrotrophic proteolytic bacteria in raw milk and unknown heat-resistant proteases with high proteolytic potential, this research aimed the assessment of psychrotrophic proteolytic microbiota predominant in milk samples from Brazil, which may reduce shelf life of dairy products. Furthermore, since none of the tests commonly used by the fluid milk industry to screen raw milk has the ability to predict the quality of heat-treated milk (Martin et al., 2011), a method based on the detection of heat-resistant proteases by an assay in microtiter plates using biotinylated casein as a substrate has been evaluated.

This work consists of three chapters. The results described in the first chapter showed that *Serratia* and *Pseudomonas* are the predominant milk spoilers in cold raw milk. The heat-resistant protease from *Pseudomonas* is well

characterized in the literature. However, there was no information about heat-resistant protease from *Serratia liquefaciens*. Thus, the second part of this work highlighted that protease from *Serratia*, which may contribute to milk spoilage, should be considered for developing a method to detect heat-resistant proteases in milk. The method developed in this research, which is described in the Chapter 3, is based on the detection of total proteolytic activity of milk samples. This method would be useful to predict the quality and the shelf life of dairy products.

References

Baglinière, F., Matéos, A., Tanguy, G., Jardin, J., Briard, V., Rousseau, F., Robert, B., Beaucher, E., Gaillard, J.L., Amiel, C., Humbert, G., Dary, A., Gaucheron, F., 2012. Quantitative and qualitative variability of the caseinolytic potential of different strains of *Pseudomonas fluorescens*: Implications for the stability of casein micelles of UHT milks during their storage. Food Chemintry, 135, 2593–2603.

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

Beyer, W., Mukendi, F.M., Kimmig, P., Böhm, R., 1998. Suitability of repetitive-DNA-sequence-based PCR fingerprinting for characterizing epidemic isolates of *Salmonella enterica* serovar Saintpaul. Journal of Clinical Microbiology, 36, 1549–1554.

Brasil, 2011. Regulamento Técnico de Produção, Identidade e Qualidade do Leite tipo A, Regulamento Técnico de Identidade e Qualidade de Leite Cru Refrigerado, Regulamento Técnico de Identidade e Qualidade de Leite Pasteurizado e Regulamento Técnico da Coleta de Leite C. Instrução Normativa n° 62, 29 dezembro 2011.

Cardoso, R.R., 2006. Influência da microbiota psicrotrófica no rendimento de queijo Minas Frescal elaborado com leite estocado sob refrigeração. Pós-graduação em Microbiologia Agrícola. Universidade Federal de Viçosa, Viçosa.

Catanio, F.S., Inay, O.M., Silva, A.S., Pereira, J.R., Tamanini, R., Beloti, V., Costa, M. de R., Souza, C.H.B., Aragon-Alegro, L.C., Santana, E.H.W., 2012. Refrigerated raw milk quality of a processing plant in the north of Parana after the implementation of changes imposed by NI 62 of 2011. Semina Ciências Agrárias, 33, 3171–3180.

Celestino, E.L., Iyer, M., Roginski, H., 1997. Reconstituted UHT-treated milk: Effects of raw milk, powder quality and storage conditions of UHT milk on its physico-chemical attributes and flavour. International Dairy Journal, 7, 129–140.

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

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

Dogan, B., Boor, K.J., 2003. Genetic diversity and spoilage potentials among *Pseudomonas* spp. isolated from fluid milk products and dairy processing plants. Applied Environmental Microbiology, 69, 130–138.

Dufour, D., Nicodème, M., Perrin, C., Driou, A., Brusseaux, E., Humbert, G., Gaillard, J.-L., Dary, A., 2008. Molecular typing of industrial strains of *Pseudomonas* spp. isolated from milk and genetical and biochemical characterization of an extracellular protease produced by one of them. International Journal of Food Microbiology, 125, 188–196.

Dupont, D., Lugand, D., Rolet-Repecaud, O., Degelaen, J., 2007. ELISA to detect proteolysis of ultrahigh-temperature milk upon storage. Journal of Agricultural and Food Chemistry, 55, 6857–6862.

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

Liao, C.-H., McCallus, D.E., 1998. Biochemical and genetic characterization of an extracellular protease from *Pseudomonas fluorescens* CY091. Applied Environmental Microbiology, 64, 914–921.

Machado, A.D.S., 2006. Atividade proteolítica de *Pseudomonas fluorescens* em biofilmes e detecção das células por anti-soro policlonal. Pós-graduação em Microbiologia Agrícola, Universidade Federal de Viçosa, Viçosa.

Machado, S.G., Bazzolli, D.M.S., Vanetti, M.C.D., 2013. Development of a PCR method for detecting proteolytic psychrotrophic bacteria in raw milk. International Dairy Journal, 29, 8–14.

Malacarne, M., Summer, A., Franceschi, P., Formaggioni, P., Pecorari, M., Panari, G., Vecchia, P., Sandri, S., Fossa, E., Scotti, C., Mariani, P., 2013. Effects of storage conditions on physico-chemical characteristics, salt equilibria, processing properties and microbial development of raw milk. International Dairy Journal, 29, 36-41.

Marchand, S., Coudijzer, K., Heyndrickx, M., Dewettinck, K., De Block, J., 2008. Selective determination of the heat-resistant proteolytic activity of bacterial origin in raw milk. International Dairy Journal, 18, 514–519.

Marchand, S., Heylen, K., Messens, W., Coudijzer, K., De Vos, P., Dewettinck, K., Herman, L., De Block, J., Heyndrickx, M., 2009a. Seasonal influence on heat-resistant proteolytic capacity of *Pseudomonas lundensis* and *Pseudomonas fragi*,

predominant milk spoilers isolated from Belgian raw milk samples. Environmental Microbiology, 11, 467–482.

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

Martin, N.H., Ranieri, M.L., Murphy, S.C., Ralyea, R.D., Wiedmann, M., Boor, K.J., 2011. Results from raw milk microbiological tests do not predict the shelf-life performance of commercially pasteurized fluid milk. Journal of Dairy Science, 94, 1211–1222.

Martins, M.L., de Araújo, E.F., Mantovani, H.C., Moraes, C.A., Vanetti, M.C.D., 2005. Detection of the *apr* gene in proteolytic psychrotrophic bacteria isolated from refrigerated raw milk. International Journal of Food Microbiology, 102, 203–211.

Matta, H., Punj, V., Kanwar, S.S., 1997. An immuno-dot blot assay for detection of thermostable protease from *Pseudomonas* sp. AFT-36 of dairy origin. Letters in Applied Microbiology, 25, 300–302.

McCarthy, C.N., Woods, R.G., Beacham, I.R., 2004. Regulation of the *aprX-lipA* operon of *Pseudomonas fluorescens* B52: differential regulation of the proximal and distal genes, encoding protease and lipase, by *ompR-envZ*. FEMS Microbiology Letters, 241, 243–248.

Munsch-Alatossava, P., Alatossava, T., 2006. Phenotypic characterization of raw milk-associated psychrotrophic bacteria. Microbiology Research, 161, 334–346.

Nabrdalik, M., Grata, K., Latala, a, 2010. Proteolytic activity of *Bacillus cereus* strains. Proceedings of ECOpole, 4, 273–277.

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

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

Pinto, C.L.O., 2004. Bactérias psicrotróficas proteolíticas do leite cru refrigerado granelizado destinado à produção do leite UHT. Pós-graduação em Microbiologia Agrícola. Universidade Federal de Viçosa, Viçosa.

Pinto, C.L.O., Machado, C.R., Cardoso, R.R., Alves, R.M., Vanetti, M.C.D., 2014. Proteolytic potential of *Pseudomonas fluorescens* isolated from refrigerated raw milk, Brazilian Journal of Sustainable Agriculture, 4, 16-25.

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

Rasolofo, E.A., St-Gelais, D., LaPointe, G., Roy, D., 2010. Molecular analysis of bacterial population structure and dynamics during cold storage of untreated and treated milk. International Journal of Food Microbiology, 138, 108–118.

Samaržija, D., 2012. Psychrotrophic bacteria and their negative effects on milk and dairy products quality. Mljekarstvo, 62, 77–95.

Sørhaug, T., Stepaniak, L., 1997. Psychrotrophs and their enzymes in milk and dairy products: Quality aspects. Trends in Food Science and Technology, 8, 35–41.

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

Tremonte, P., Tipaldi, L., Succi, M., Pannella, G., Falasca, L., Capilongo, V., Coppola, R., Sorrentino, E., 2014. Raw milk from vending machines: Effects of boiling, microwave treatment, and refrigeration on microbiological quality. Journal of Dairy Science, 97, 3314–3320.

Vithanage, N.R., Yeager, T.R., Jadhav, S.R., Palombo, E.A., Datta, N., 2015. Comparison of identification systems for psychrotrophic bacteria isolated from raw bovine milk. International Journal of Food Microbiology, 189, 26-38.

Woods, R.G., Burger, M., Beven, C.-A., Beacham, I.R., 2001. The *aprX-lipA* operon of *Pseudomonas fluorescens* B52: a molecular analysis of metalloprotease and lipase production. Microbiology, 147, 345–354.

Zhang, S., Li, H., Uluko, H., Liu, L., Pang, X., Lv, J., 2015. Investigation of protease production by *Pseudomonas fluorescens* BJ-10 and degradation on milk proteins. Journal of Food Processing and Preservation, *In press*.

CHAPTER 1 - *Pseudomonas* spp. and *Serratia liquefaciens* as predominant spoilers in cold raw milk

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Abstract: The storage of fresh raw milk at low temperature does not prevent proliferation of psychrotrophic bacteria that can produce heat-resistant proteolytic enzymes contributing to the reduced shelf life of dairy products. This study aimed to identify the dominant psychrotrophic proteolytic enzyme-producing population of raw milk from Brazil. Raw milk samples collected in three different cooling tanks in Brazil were stored at optimal (45 h at 4 °C following by 3 h at 7 °C) and suboptimal (45 h at 7 °C following by 3 h at 10 °C) conditions to simulate farm storage and transportation allowed by Brazilian laws. The highly proteolytic enzyme-producing strains isolated from stored cold raw milk were characterized by repetitive sequence-based PCR analysis. This clustering resulted in eight different clusters and four solitary fingerprints. The most proteolytic isolates from each rep-cluster were selected for identification using miniaturized kit, 16S rDNA and *rpoB* gene sequencing. *Serratia liquefaciens* (73.9 %) and *Pseudomonas* spp. (26.1 %) were identified as the dominant psychrotrophic microorganisms with high spoilage potential. The knowledge of milk spoilage microbiota will contribute to improved quality of milk and dairy products.

Keywords: heat-resistant proteolytic enzyme, psychrotrophic, proteolytic, Rep-PCR, spoilage

Practical Application: This study evaluated the dominant psychrotrophic microbiota linked to milk spoilage. The results highlighted storage conditions of raw milk have an impact on shelf life of dairy products. Furthermore, this work demonstrated that *S. liquefaciens* isolates from raw milk produces heat resistant proteolytic enzymes, which may lead to spoilage of raw milk and milk products.

1.1. Introduction

The microbial contamination of raw milk at the farm level occurs via bacterial contamination from the external surface of cow's udder and teats, from the surface of milking equipment or from mastitis organisms from the udder (Murphy and Boor, 2000). Usually, raw milk is stored at low temperatures to minimize microbial growth. Storage of fresh milk at cold temperatures was implemented in Brazil in the second half of the 90s. According to the Brazilian legislation (Brasil, 2011), raw milk should be stored at temperatures below 4 °C in a cooling tank or at a temperature limit of 7 °C in a water bath to a maximum time of 3 hours after milking. Moreover, this same law concerns the transportation of milk to the industrial plant, which must be done in isothermal tank car with the maximum temperature of 10 °C in a maximum time interval of 48 hours after the milking. These storage conditions differ in the European Union (Council Directives, 1992; Regulation, 2004) where raw milk must be cooled to a temperature of 8 °C or lower in the case of daily collection or 6 °C or lower if collection is not daily.

The cold storage for long periods supports the growth of psychrotrophic microorganisms. Many psychrotrophic microorganisms are known as proteolytic and/or lipolytic enzymes producers (Marchand et al., 2009b; Sørhaug and Stepaniak, 1997). Although, psychrotrophic bacteria are inactivated by heat treatments such as pasteurization and ultra-high temperature processing (UHT), proteolytic and lipolytic enzymes produced by this group of microorganisms can keep their spoilage potential even after heating (Baglinière et al., 2013; Champagne et al., 1994). Proteolysis in milk can cause several technological problems such as increase of viscosity, age gelation and bitter flavor in milk and dairy products and reduction of yield in cheese manufacturing (Datta and Deeth, 2001; García-Risco et al., 1999; Topçu et al., 2006; Valero et al., 2001).

Several studies have been conducted to characterize the psychrotrophic microbiota in milk and to identify the predominant microorganisms after cold storage (Dogan and Boor, 2003; Ercolini et al., 2009; Hantis-Zacharov and Halpern, 2007; Marchand et al., 2009a; Raats et al., 2011; Rasolofo et al., 2010; Tremonte et al., 2014). Hantis-Zacharov and Halpern (2007) and Raats and others (2011) reported, using culture-dependent and molecular methods, *Pseudomonas*, *Acinetobacter* and actinobacteria are the dominant groups in Israeli refrigerated milk. *Pseudomonas* genus was also considered the predominant psychrotrophic group with high spoilage potential in America (Dogan and Boor, 2003), Belgium (Marchand et al., 2009a), Canada (Rasolofo et al., 2010) and Italy (Ercolini et al., 2009; Tremonte et al., 2014). However, other psychotrophic species such as *Serratia marcescens* and *Serratia liquefaciens*, which may secrete protease, have been identified along with *Pseudomonas* (Decimo et al, 2014; Teh et al., 2011).

Nörnberg and others (2010) demonstrated *Burkholderia cepacia*, *Klebsiella oxytoca* and *Aeromonas* sp. are the most proteolytic enzyme-producing group found in Brazilian raw milk samples stored in cold temperature. Nevertheless, molecular methods were not used to check and supplement the identification. Although, Martins and others (2006) have used random amplified polymorphic DNA (RAPD) fingerprinting technique to assess the genetic diversity of Gram-negative proteolytic enzyme-producing psychrotrophic bacteria isolated from Brazilian refrigerated raw milk, the study was not focused on the dominant psychrotrophic microbiota with high spoilage potential.

A specific study towards predominant psychrotrophic microbiota producing heat-resistant proteolytic enzymes in cold raw milk may contribute to knowledge of milk spoilers and to development of alternative and rapid methods to predict shelf life of heat-treated dairy products. The goal of this research was to simulate cold storage on farms and to identify the dominant psychrotrophic microbiota with highest spoilage potential using a molecular approach in addition to the classic phenotypic method.

1.2. Materials and methods

1.2.1. Sampling

Three composite samples of raw milk were collected in April 2013 (Samples A, B and C). Each sample corresponded to a mixture of raw milk obtained from three farms located within a radius of 50 km from Viçosa, Minas Gerais, Brazil. Each sample was collected aseptically from a cooling tank (2000 L) immediately after the last neighboring farmer had placed his production in the tank. The farms considered in this study participate in the Development Program of Dairy Cattle (PDPL) – Universidade Federal de Viçosa, which provides technical assistance to small farmers with a view to the growth of the dairy farming and increased competitiveness, due to the better quality of milk. Farmers had an average of 20 milk-producing cows. The average time from first milking to sampling never exceeded 90 minutes. The samples (600 mL) were transported on ice to the laboratory.

1.2.2. Simulation of cold storage

Based on Brazilian law (Brasil, 2011), time and temperature simulating optimal (I) and suboptimal (II) conditions for farm storage and transportation of milk before industrial processing were chosen (Table 1). To simulate the milking peaks, the samples were heated twice a day, in the morning and in the afternoon (Table 1).

Table 1 - Simulation of the dairy chain from farm milk tank and transportation on a 0.6 L scale.

Simulating storage mode	Condition I (Optimal)		Condition II (Suboptimal)	
	Temperature (°C)	Storage time (h)	Temperature (°C)	Storage time (h)
Farm milk tank (with stirring[a])	4	45	7	45
Milking Peak[b]	7		10	
Transport (without stirring)	7	3	10	3

[a] Stirring at 100 rpm.

[b] To simulate the warming up of the tank milk when the fresh milk enters the tank. Once milk temperature reached the milking peak, samples were cooled down to tank temperature. It took, approximately, 90 min.

Each sample (A, B and C) corresponding to an independent simulation was divided in two equal portions. One portion was incubated in optimal conditions and the other, in suboptimal conditions (Table 1). Thus, three independent simulations (Samples A, B and C) using two different conditions (optimal and suboptimal) were performed.

1.2.3. Microbial analysis

Composite milk samples A, B and C from three different cooling tanks were collected and analyzed immediately before the beginning of the simulation (S1 = t_0), after 8 h (S2 = t_0 + 8 h), 24 h (S3 = t_0 + 24 h), 42 h (S4 = t_0 + 42 h), 45 h (S5 t_0 + 45 h) and 48 h (S6 = t_0 + 48 h) of storage. The total aerobic, proteolytic enzyme-producing psychrotrophic bacteria and presumptive *Pseudomonas* counts of each sample were determined. The total aerobic plate counting was established by spread plating of serial dilutions on plate count agar - PCA (HiMedia, Mumbai, India) with incubation at 30°C for 2 days (ISO, 2003). The proteolytic enzyme-producing psychrotrophic bacteria counting was performed on PCA supplemented with skim milk 2 % (w/v) with incubation at 6.5 ± 0.5 °C for 10 days (IDF, 1991). The presumptive *Pseudomonas* counting was accomplished on *Pseudomonas* agar base (16 g L^{-1} gelatin peptone, 10 g L^{-1} casein peptone, 10 g L^{-1} potassium sulphate, 1.4 g L^{-1} magnesium chloride, 11 g L^{-1} agar, 10 g L^{-1} lactose and 0.02 g L^{-1} bromothymol blue) supplemented with CFC (10 mg L^{-1} cetrimide, 10 mg L^{-1} fucidin and 50 mg L^{-1} cephalosporin) selective agent (HiMedia, Mumbai, India) with incubation at 25 °C for 2 days. All components of *Pseudomonas* agar base were purchased from Merck KGaA (Darmstadt, Germany). The microbial analysis were performed in duplicate.

1.2.4. Isolation of proteolytic psychrotrophic bacteria

Based on the presence of a clear zone around colonies, proteolytic enzyme-producing psychrotrophic bacteria with different colony morphologies were picked using needles from PCA supplemented with skim milk 2% at the beginning (S1), at the middle (S3), at the end (S5) of the farm storage simulation and at the end of transportation simulation (S6). An average of 10 % of the total amount of colonies was picked up at each isolation point. Colonies were isolated by streaking on PCA

supplemented with skim milk 2 % (w/v). The isolate colonies were inoculated in Brain Heart Infusion Broth - BHI (HiMedia, Mumbai, India) and incubated at 25 °C for 24 h. Subsequently, pure cultures were stored at -80 °C under cryoprotection (glycerol 20 % v/v).

1.2.5. Screening of isolates producing heat-stable proteolytic enzymes

Cryopreserved isolates were recovered in BHI, at 25 °C until growth was visually present. Then, the isolates were inoculated in PCA supplemented with skim milk 4 % (w/v). The plates were incubated at 6.5 ± 0.5 °C and the diameter of the clear halo around colonies indicating proteolytic activity was measured after 10 days of incubation. The isolates that presented a diameter of the halo larger than the average (8.3 mm) were considered highly proteolytic (De Jonghe et al., 2011).

The highly proteolytic isolates were inoculated in 10 mL of reconstituted skim milk powder 12 % (w/v) and incubated overnight at 25 °C. Milk-grown isolates were serially diluted and inoculated in 10 mL of skim milk 12 % at a final concentration of approximately 10^3 CFU mL^{-1}. Inoculated milk samples were stored at 7 °C for 4 days. The milk samples were heated at 95 °C for 8 min and 45 s as described by Marchand and others (2008). The heated milk samples were stored at 37 °C for 2 weeks. For each sample, a duplicate was stored as control at -20 °C. Hydrolysis of proteins was measured by the trinitrobenzenesulfonic acid (TNBS) method as described by Marchand and others (2008).

1.2.6. Typing and identification of isolates producing heat-stable proteolytic enzymes

1.2.6.1. Rep-PCR

The highly heat-stable proteolytic enzyme-producing isolates were grown on BHI broth and the DNA extraction were performed using the Wizard® Genomic DNA Purification kit (Promega, Madison, USA). DNA quality was analyzed by electrophoresis on a 0.8% (w/v) agarose gel and by spectrophotometry based on the A260/A280 ratio. These isolates were characterized by repetitive sequence-based PCR analysis with $(GTG)_5$ primer as described by Marchand and others (2009a). The fingerprints were analyzed using the Bionumerics 6.0 software package (Applied Maths, Sint-Martens-Latem, Belgium) to construct dendrograms by the

Unweighted Pair Group Method with Arithmetic Mean (UPGMA method) using the Pearson correlation coefficient.

1.2.6.2. Identification

The most proteolytic isolates from each rep-cluster were Gram-stained, examined for cell shape, oxidase and catalase activity and identified using API20 kits (bioMerieux, Marcy l'Etoile, France) and sequencing.

The 16S rDNA and *rpoB* gene amplification was performed using primers and PCR conditions described by Marchand and others (2009a). The PCR products were sequenced at Macrogen (Seoul, South Korea). The sequences were aligned with sequences retrieved from GenBank using Basic Local Alignment Tool (BLAST). The identification of *Pseudomonas fluorescens* was checked using species-specific primers to amplify a conserved region of 16S rDNA gene among strains belonging this species as described by Scarpellini and others (2004).

1.2.7. Statistical analysis

Statistical analysis was performed on log of bacterial counts using base 10. Since the samples were taken subsequently from the same batch at six time intervals (0, 18, 24, 42, 45 and 48 h), the measurements are likely to be highly correlated. Therefore, generalized estimating equations (GEE) were performed using GENMOD procedure in SAS 9.1.3 (SAS Institute, 2003). The explanatory variables were bacteria, condition (I and II) and time. The correlation structure used was autoregressive AR(1) because we expect repeated measures that are strongly correlated when close together in time.

1.3. Results

1.3.1. Microbial succession during storage in raw milk samples

A total aerobic plate count of 7.9, 5.1 and 6.9 log CFU mL^{-1} was obtained for composite samples A, B and C, respectively, at the beginning of the storage (S1) with an arithmetical average of 6.7 log CFU mL^{-1}. At the end of storage (S6), the arithmetical average of total aerobic plate count for the three samples was 7.0 and 8.1 log CFU mL^{-1} in conditions I and II (Table 1), respectively (Figure 1A).

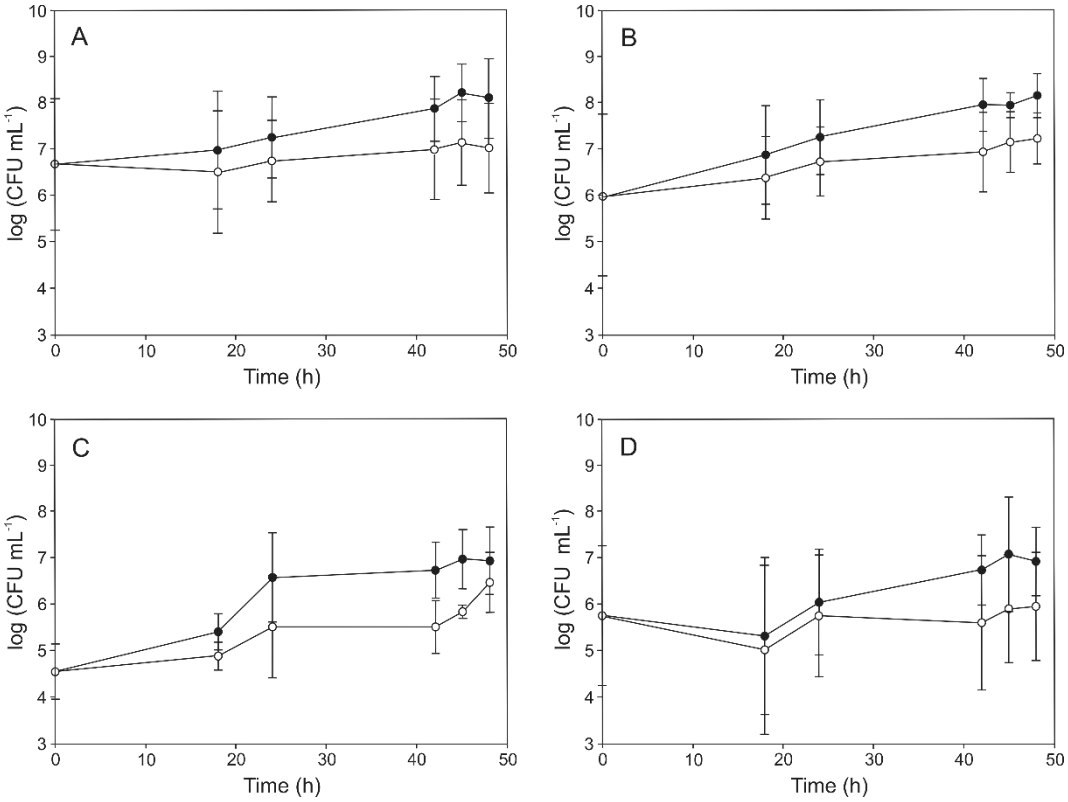

Figure 1 – Total aerobic plate count (A), total psychrotrophic plate count (B), total proteolytic psychrotrophic plate count (C) and total *Pseudomonas* plate count (D) in raw milk samples during the simulated cold storage in condition I (○) and condition II (●). Error bars correspond to standard deviation.

There were significant main effects of bacteria, condition and time ($P<0.001$) and significant interaction between condition and time ($P<0.05$), indicating that differences in bacterial counts between the two conditions varied with time. Regarding the source of variation bacteria separately, the counts of mesophilic and psychrotrophic bacteria did not differ statistically (95% confidence interval (CI) 7.0-7.3 and 7.0-7.1 log CFU mL^{-1}, respectively) and were significantly ($P<0.0001$) higher compared with count of *Pseudomonas* and proteolytic psychrotrophic bacteria (95% CI 5.7-6.3 and 5.4-6.2 log CFU mL^{-1}, respectively) (Table 2).

The count of mesophilic bacteria did not differ statistically ($P>0.05$) under optimal storage condition (45 h at 4 °C following by 3 h at 7 °C), but became statistically significant ($P<0.01$) from S4 onwards under suboptimal storage condition (45 h at 7 °C following by 3 h at 10 °C) (Table 2). Similar result was found for *Pseudomonas*. The count of psychrotrophic bacteria became statistically

significant (P<0.05) from S4 onwards under optimal storage condition and from S2 onwards under suboptimal storage condition. Similar result was found for proteolytic enzyme-producing psychrotrophic bacteria (Table 2).

Table 2 – Estimates of parameters from generalized estimating equations (GEE) analysis of bacterial count (log CFU mL^{-1}) after six times (from S1 to S6) and two storage conditions (Conditions I and II) in raw milk samples.

Effect	Estimate	Standard Error	*p*-value	95% CI
Bacteria			<.0001	
Mesophilic	7.18	0.066		7.052-7.312
Psychrotrophic	7.06	0.018		7.027-7.097
Pseudomonas	5.99	0.148		5.706-6.286
Proteolytic enzyme-producing psychrotrophic	5.83	0.205		5.429-6.233
Bacteria/Condition/Time			<.0001	
Mesophilic/I[a]/S1[b]	6.67	0.320		6.046-7.300
Mesophilic/I/S6	7.03	0.119		6.797-7.263
Mesophilic/II/S1	6.67	0.320		6.046-7.300
Mesophilic/II/S4	7.87	0.172		7.533-8.207
Pseudomonas/I/S1	5.76	0.385		5.009-6.518
Pseudomonas/I/S6	5.97	0.222		5.532-6.401
Pseudomonas/II/S1	5.76	0.385		5.009-6.518
Pseudomonas/II/S4	6.75	0.038		6.671-6.822
Psychrotrophic/I/S1	5.99	0.450		5.108-6.872
Psychrotrophic/I/S5	7.17	0.085		7.003-7.337
Psychrotrophic/II/S1	5.99	0.450		5.108-6.872
Psychrotrophic/II/S3	7.27	0.006		7.257-7.283
Proteolytic enzyme-producing psychrotrophic/I/S1	4.56	0.410		3.756-5.364
Proteolytic enzyme-producing psychrotrophic/I/S5	6.46	0.560		5.368-7.565
Proteolytic enzyme-producing psychrotrophic/II/S1	4.56	0.410		3.756-5.364
Proteolytic enzyme-producing psychrotrophic/II/S3	6.58	0.109		6.362-6.791

[a] Condition I is 45 h incubation at 4 °C following by 3 h at 7 °C and Condition II is 45 h incubation at 7 °C following by 3 h at 10 °C.
[b] Six simulation times: S1 = t_0, S2 = t_0 + 8 h, S3 = t_0 + 24 h, S4 = t_0 + 42 h, S5 t_0 + 45 h and S6 = t_0 + 48 h.

The milk refrigeration in condition II did not inhibit the growth of proteolytic enzyme-producing psychrotrophic bacteria and *Pseudomonas*, which reached 6.9 ± 0.6 log CFU mL^{-1} after 48 h cold storage whereas the initial concentration was 4.6 ± 0.6 and 5.8 ± 1.2 log CFU mL^{-1}, respectively (Figure 1C and 1D). However, when lower temperatures were used during cold storage simulation (condition I), the *Pseudomonas* concentration remained at 6.0 ± 1.2 log CFU mL^{-1} (Figure 1D).

1.3.2. Spoilage potential of isolates

Based on colony morphology and the presence of a clear zone on the PCA supplemented with skim milk 2 % (w/v), 150 isolates, which were able to hydrolyze the casein, were isolated, purified and stored at – 80 °C.

The spoilage potential of the 150 microorganisms isolated from raw milk during the storage in both conditions (I and II) is depicted in Figure 2. The strains isolated after 45 (S5) and 48 h (S6) storage exhibited a lower dispersion of spoilage potential (illustrated by shorter length bars in Figure 2) than the strains isolated immediately after milking (S1) and after 24 h (S3) simulation. The lower variability of spoilage potential of isolates after 45 and 48 h storage (S5 and S6) indicated proteolytic enzyme-producing strains were selected during cold storage simulation.

A total of 115 isolates (76.6 % of proteolytic enzyme-producing psychrotrophic isolates), presented a diameter of clear zone bigger than the average (8.3 mm) and were considered highly proteolytic. All these highly proteolytic isolates were evaluated for heat-resistant proteolytic activity and the ability to produce heat-resistant proteolytic enzymes was assumed if the average of two tests was above 0.2 µmol of glycin equivalents mL^{-1} (Marchand et al., 2009a). Sixty-nine strains (60 % of highly proteolytic isolates) displayed heat-resistant proteolytic activity. At the beginning (S1) of cold storage, 13.6 % of proteolytic enzyme-producing psychrotrophic isolates were heat-resistant proteolytic enzymes producers. While after 24 (S3), 42 (S5) and 45 (S6) h of cold storage, the heat-resistant proteolytic enzymes producers represented 34.7 %, 56.3 % and 71.0 %, respectively. The selection of isolates with greater spoilage potential during the raw milk cold storage could also be observed when isolates were evaluated for the ability to produce heat-resistant proteolytic enzymes.

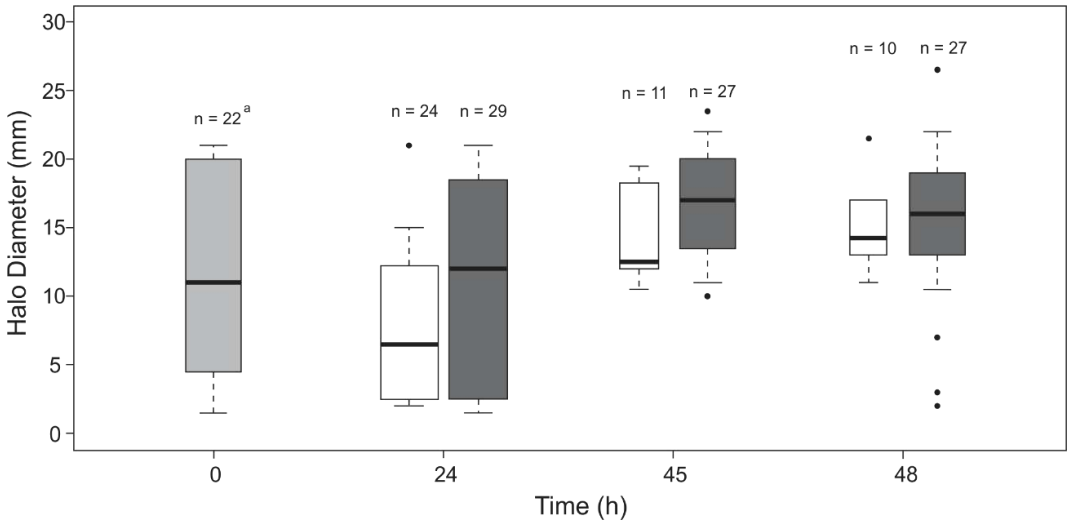

Figure 2 – Spoilage potential of strains isolated during the raw milk storage in different conditions of storage (Condition I (white); Condition II (dark gray)). The results are shown with the numbers of isolates tested (n). The solid line within the box marks the median. The boundaries of the box represent 25th and 75th percentiles. Whiskers above and below the box indicate the 10th and 90th percentiles. The outliers are shown as dots.

[a] There is only one bar at the beginning of storage (S1- time 0), because the original sample was the same for both conditions.

1.3.3. Characterization and identification of heat-resistant proteolytic enzymes producers

All 69 heat-resistant proteolytic enzymes producers were subjected to $(GTG)_5$-PCR fingerprint analysis resulting in eight different clusters with similarity indices of at least 70 % and four solitary fingerprints (Figure 3). One major cluster (I) was found, encompassing 41.6 % of the isolates, which exhibited a mean value of 1.07 µmol glycin equivalent mL^{-1}. Although cluster I is a major cluster considering number of isolates, the clusters II and III, containing 11.1 and 5.5 % of isolates, displayed a mean value of 3.83 and 2.59 µmol glycin equivalent mL^{-1} respectively.

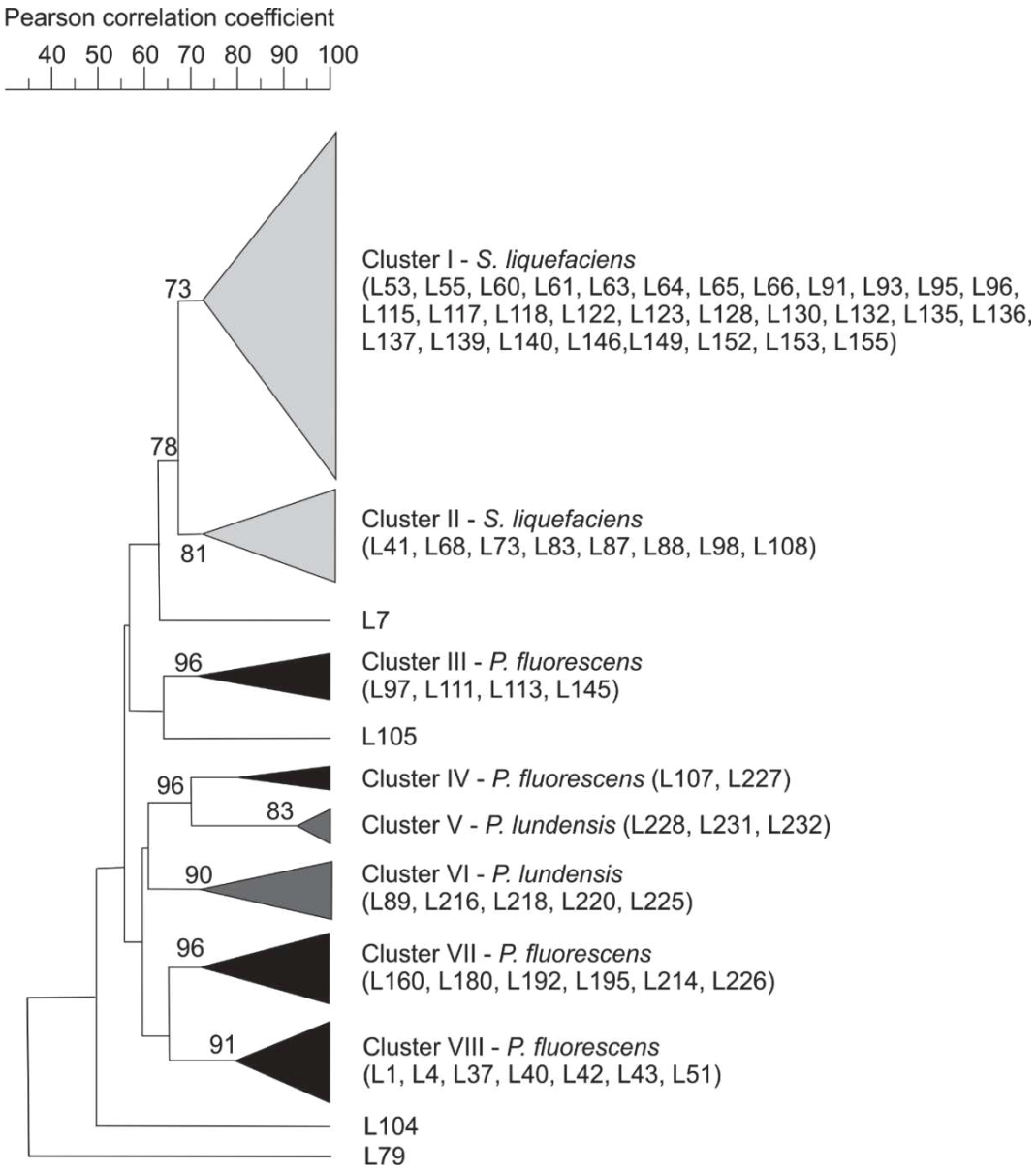

Figure 3 – Fingerprints cluster analysis of heat-resistant proteolytic enzyme producers. The fingerprinting patterns were constructed using the unweighted pair-group (UPGMA) method. The cophenetic correlation coefficient is a measure of how faithfully a dendrogram preserves the pairwise distances between the original unmodeled data points. Only the cophenetic correlation coefficients above 70 % are shown.

At least 12.5 % of isolates from each cluster and two solitary fingerprint isolates, in total 23 selected cultures, were characterized and identified. All isolates were found to be Gram-negative, rod-shaped and catalase positive. The oxidase-negative isolates (73.9 %) were characterized with API20E and identified as

Serratia liquefaciens (17 isolates). The oxidase-positive isolates (26.1 %) were identified as *Pseudomonas* sp. with four isolates as *P. fluorescens* and two isolates as *Pseudomonas putida* using API20NE (Table 3). As the API database is not complete, the sequences of *rpoB* and 16S rDNA genes of all 23 isolates were compared with GenBank (NCBI). The 16S rDNA gene sequencing could only show that the 17 oxidase-negative isolates were members of the *Serratia* genus and the other six oxidase-positive isolates were members of the *Pseudomonas* genus.

Table 3 – Polyphasic identification of isolates with heat-resistant proteolytic activity.

$(GTG)_5$-REP-PCR cluster	Proteolytic activity (µmol glicine/mL)	API Identification (% similarity)	rpoB identification (based on BLASTn result of *rpoB* gene sequences)	Specific PCR targeting 16S rDNA gene for the presence of *P. fluorescens*
I	0.448 – 3.783	75.6 % *S. liquefaciens*	99 - 100 % *S. liquefaciens* CCM2716 (JX425336)	-
II	0.925 – 13.757	75.6 % *S. liquefaciens*	99 % *S. liquefaciens* ATCC27592 (CP006253)	-
III	0.246 – 7.889	99.7 % *P. fluorescens*	97 % *P. fluorescens* 13-1F1 (HM234519)	-
IV	0.829 – 1.57	99.7 % *P. fluorescens*	98 % *P. fluorescens* LMG 14576 (HE586416)	+
V	0.389 – 1.153	99.1 % *P. putida*	99 % *P. lundensis* CIP103272T (AJ717428)	-
VI	0.304 – 0.563	99.1 % *P. putida*	99 % *P. lundensis* CIP103272T (AJ717428)	-
VII	0.285 – 1.109	99.7 % *P. fluorescens*	99 % *P. fluorescens* LMG 14562 (HE586410)	+
VIII	0.231 – 0.415	92.5 % *P. fluorescens*	99 % *P. fluorescens* 156-2F4 (HM234584)	-

The *rpoB* gene sequencing was performed for higher-resolution identification (Deperrois-Lafarge and Meheut, 2012; Marchand et al., 2009a). The 17 isolates belonging to the clusters I and II were identified as *S. liquefaciens* confirming API identification. However, the isolates identified as *P. putida* using API kit were identified as *Pseudomonas lundensis* based on *rpoB* gene sequences (Table 3). The identification of isolates from clusters IV and VII as *P. fluorescens* by sequencing and API test was confirmed when the amplification of 16S rDNA gene was performed using species-specific primers, however, this was not the case for isolates belonging to the clusters III and VIII.

1.4. Discussion

Microbial diversity in raw milk has been targeted in several studies (Delbès et al., 2007; Ercolini et al., 2009; Giannino et al., 2009; Martins et al., 2006; Raats et al., 2011; Rasolofo et al., 2010) as well as the effect of cold storage on bacterial community and raw milk quality (De Jonghe et al., 2011; Gargouri et al., 2013; Izidoro et al., 2013; Kumaresan et al., 2007; Perin et al., 2012; Wiking et al., 2002). However, the goal of this study, contrary to the majority of the previous ones, was to assess the Brazilian raw milk quality and to identify the predominant psychrotrophic heat-resistant proteolytic enzymes producers during cold storage using molecular methods in addition to miniaturized phenotypic kits. All studies that assessed the quality of Brazilian raw milk (Izidoro et al., 2013; Nörnberg et al., 2010; Perin et al., 2012) including this study collected samples from small areas around the country in different seasons, which may have influenced the results.

The rate of microbial contamination in raw milk is influenced by bacterial contamination on external surface of udder and teats, on surface of equipment and mastitis-causing organisms during milking (Murphy and Boor, 2000). According to European and Brazilian microbiological requirements described in Council Directive 92/46/EEC (Council Directives, 1992), Regulation (EC) No 853/2004 (Regulation, 2004) and IN n° 62 (Brasil, 2011), raw milk intended for heat-treated drinking milk production must not exceed 1.0×10^5 and 3.0×10^5 CFU mL^{-1} of total aerobic plate count, respectively. The total aerobic plate count of raw milk in this study exceeded the regulatory limit established by the European Union at the beginning of cold storage. Only sample B, which presented an initial aerobic plate count of 1.4×10^5 CFU mL^{-1}, exhibited an acceptable bacteriological quality according to Brazilian standards. However, after 18 h storage, the European and Brazilian limit values were already exceeded in both storage conditions (Figure 1A) indicating poor hygienic conditions in the milking steps.

Although there is no official requirement for psychrotrophic count in raw milk, Vylětelová and others (1999) reported a population of 4.5×10^4 CFU mL^{-1} for psychrotrophic proteolytic and/or lipolytic enzyme-producing bacteria as a limit to produce heat-processed dairy products with extended shelf life. Considering this limit, proteolytic enzyme-producing psychrotrophic count in our milk samples exceeded this limit after 18 h cold storage (Figure 1C), which shows that even under

Brazilian regulation and recommendation of storing milk, dairy products quality can be compromised by improper hygienic conditions of milk farms. If good milking practices are not followed properly, raw milk contamination can exceed official or empirical requirements despite being kept in lower cold temperatures.

Psychrotrophic microorganisms may produce heat-resistant enzymes, which lead to off-flavor in dairy products (Sørhaug and Stepaniak, 1997). These enzymes can resist the heat-treatment and spoil dairy products during storage, thus, reducing their shelf life. Although, *Pseudomonas* is the predominant proteolytic enzymes producer genus found in refrigerated raw milk samples (Dogan and Boor, 2003; Marchand et al., 2009a; Raats et al., 2011; Rasolofo et al., 2010), *Serratia liquefaciens* was, surprisingly, one of the most prevalent psychrotrophic species with ability to produce heat-resistant proteolytic enzymes in Brazilian raw milk rather than *B. cepacia*, *K. oxytoca* and *Aeromonas* spp. highlighted by Nörnberg and others (2010).

Serratia spp. were also detected in raw milk samples stored at 4 °C for 24 h (Lafarge et al., 2004), in bulk tank milk (Decimo et al., 2014) and in a milk processing plant (Cleto et al., 2012), but these authors did not evaluate the capacity of heat-resistant proteolytic enzymes production of *Serratia* isolates. Teh and others (2011), in addition to isolating *S. liquefaciens* from internal surfaces of raw milk road tankers, also showed that this species may produce heat-resistant proteolytic enzymes. Thus, the *Serratia* genus should be considered in the development of alternative and rapid methods for monitoring the quality of refrigerated raw milk.

Teh and others (2012) highlighted *S. liquefaciens* may produce biofilms in single culture and in co-culture with *Streptococcus uberis* in an *in vitro* model that simulates the stainless steel surfaces of a tanker after milk collection. Nevertheless, Cleto and others (2012) showed *Serratia* spp. general capacity of forming biofilm was overall much higher than for *Pseudomonas* spp. isolates. It is possible that *Serratia* isolates were found as one of the most predominant proteolytic enzymes producers because *Pseudomonas* spp. biofilms tended to have a smaller ratio of mass:cells and occur together with species, as *Serratia* spp., presenting the opposite pattern (Cleto et al., 2012). The presence of a single different strain may have a significant effect on the microbial dynamics in dairy products (Lafarge et al., 2004). The quality of UHT milk stored for 5 months was compromised when raw milk was

exposed to multispecies biofilms of *P. fluorescens*, *S. liquefaciens* and *Staphylococcus aureus* (Teh et al., 2014). Furthermore, cold storage of raw milk amplified some heat-resistant proteolytic enzyme producers (Figure 2). The emergence of some bacterial species that were barely detectable in the initial sample and the disappearance of initially major species in raw milk samples during cold storage were attested by several studies (Lafarge et al., 2004; Raats et al., 2011; Rasolofo et al., 2010).

The dynamics of a microbial population in dairy products have been assessed by molecular methods, mainly sequencing a fragment of 16S rDNA gene and comparing with public databases. However, Marchand and others (2009a) demonstrated identification based on API tests and 16S rDNA gene sequencing can result in a unreliable and inaccurate identification of isolates. Therefore, in this work, the *rpoB* gene was used for higher-resolution identification (De Jonghe et al., 2011). Using this approach, *S. liquefaciens*, *P. fluorescens* and *P. lundensis* were identified as predominant proteolytic spoilers in Brazilian raw milk.

1.5. Conclusions

The quality of the three raw milk samples was not in accordance with requirements described in the Brazilian legislation. This study showed that even if milk is kept in recommended conditions, the final product quality may be compromised by poor hygienic practices on milk farms and by milk storage at cold temperatures during long periods before processing. This reduced shelf life of dairy products may be a consequence of high proteolytic potential exhibited by the dominant psychrotrophic microbiota isolated from cold raw milk, including *S. liquefaciens*, *P. fluorescens* and *P. lundensis*.

The heat-resistant protease from *Pseudomonas* genus, AprX, which is often related to milk spoilage, is well described in the literature. However, as far as we know, the heat-resistant protease produced by *S. liquefaciens* was not characterized neither the encoding gene of this enzyme was identified. The proteases from *S. liquefaciens* were compared to the heat-resistant protease from *Pseudomonas* in the Chapter 2 to confirm if *S. liquefaciens* may contribute to milk spoilage.

1.6. References

Baglinière F, Matéos A, Tanguy G, Jardin J, Briard-Bion V, Rousseau F, Robert B, Beaucher E, Gaillard JL, Amiel C, Humbert G, Dary A, Gaucheron F. 2013. Proteolysis of ultra high temperature-treated casein micelles by AprX enzyme from *Pseudomonas fluorescens* F induces their destabilisation. International Dairy Journal 31(2):55-61.

Brasil. 2011. Regulamento Técnico de Produção, Identidade e Qualidade do Leite tipo A, Regulamento Técnico de Identidade e Qualidade de Leite Cru Refrigerado, Regulamento Técnico de Identidade e Qualidade de Leite Pasteurizado e Regulamento Técnico da Coleta de Leite Cru Refrigerado e seu Transporte a Granel. Instrução Normativa nº 62, de 29 de de dezembro de 2011. Diário Oficial [da] República Federativa do Brasil, Brasília, DF: Ministério da Agricultura, Pecuária e Abastecimento. p. 6-37.

Champagne CP, Laing RR, Roy D, Mafu AA. 1994. Psychrotrophs in dairy products: their effects and their control. Critical Reviews in Food Science and Nutrition 34(1):1-30.

Cleto S, Matos S, Kluskens L, Vieira MJ. 2012. Characterization of contaminants from a sanitized milk processing plant. PLoS ONE 7(6):e40189.

Council Directives. 1992. Laying down the health rules for the production and placing on the market of raw milk, heat-treated milk and milk-based products. In: The European Parliament, editor. 92/46 EEC. Strasbourg: Official Journal of the European Communities.

Datta N, Deeth HC. 2001. Age Gelation of UHT Milk—A Review. Food and Bioproducts Processing 79(4):197-210.

De Jonghe V, Coorevits A, Van Hoorde K, Messens W, Landshoot A, De Vos P, Heyndrickx M. 2011. Influence of storage conditions on the growth of

Pseudomonas species in refrigerated raw milk. Applied and Environmental Microbiology 77(2):460-70.

Decimo M, Morandi S, Silvetti T, Brasca M. 2014. Characterization of gram-negative psychrotrophic bacteria isolated from Italian bulk tank milk. Journal of Food Science 79(10):M2081-M90.

Delbès C, Ali-Mandjee L, Montel M-C. 2007. Monitoring bacterial communities in raw milk and cheese by culture-dependent and -independent 16S rRNA gene-based analyses. Applied and Environmental Microbiology 73(6):1882–91.

Deperrois-Lafarge V, Meheut T. 2012. Use of the *rpoB* gene as an alternative to the V3 gene for the identification of spoilage and pathogenic bacteria species in milk and milk products. Letters in Applied Microbiology 55(2):99-108.

Dogan B, Boor KJ. 2003. Genetic diversity and spoilage potentials among *Pseudomonas* spp. isolated from fluid milk products and dairy processing plants. Applied and Environmental Microbiology 69(1):130-8.

Ercolini D, Russo F, Ferrocino I, Villani F. 2009. Molecular identification of mesophilic and psychrotrophic bacteria from raw cow's milk. Food Microbiology 26(2):228-31.

García-Risco MR, Ramos M, López-Fandiño R. 1999. Proteolysis, protein distribution and stability of UHT milk during storage at room temperature. Journal of the Science of Food and Agriculture 79(9):1171-8.

Gargouri A, Hamed H, ElFeki A. 2013. Analysis of raw milk quality at reception and during cold storage: combined effects of somatic cell counts and psychrotrophic bacteria on lipolysis. Journal of Food Science 78(9):M1405-M11.

Giannino ML, Marzotto M, Dellaglio F, Feligini M. 2009. Study of microbial diversity in raw milk and fresh curd used for Fontina cheese production by culture-independent methods. International Journal of Food Microbiology 130(3):188-95.

Hantis-Zacharov E, Halpern M. 2007. Culturable psychrotrophic bacterial communities in raw milk and their proteolytic and lipolytic traits. Applied and Environmental Microbiology 73(22):7162-8.

IDF. 1991. Enumeration of psychrotrophic microorganisms - Colony count at 6.5°C. In: Federation ID, editor. Standard n° 101A. Bruxellas.

ISO. 2003. Microbiology of food and animal feed - Horizontal method for the enumeration of microorganisms - Part 2: Colony count at 30 degrees C by the surface plating technique. ISO/FDIS 4833-2: BSI.

Izidoro TB, Pereira JG, Soares VM, de Almeida Nogueira Pinto JP. 2013. Effect of psychrotrophic growth on the milk fat fraction at different temperatures of storage. Journal of Food Science 11(10):1750-4.

Kumaresan G, Annalvilli R, Sivakumar K. 2007. Psychrotrophic spoilage of raw milk at different temperatures of storage. Journal of Applied Sciences Research 3(11):1383-7.

Lafarge V, Ogier J-C, Girard V, Maladen V, Leveau J-Y, Gruss A, Delacroix-Buchet A. 2004. Raw cow milk bacterial population shifts attributable to refrigeration. Applied and Environmental Microbiology 70(9):5644-50.

Marchand S, Coudijzer K, Heyndrickx M, Dewettinck K, De Block J. 2008. Selective determination of the heat-resistant proteolytic activity of bacterial origin in raw milk. International Dairy Journal 18(5):514-9.

Marchand S, Heylen K, Messens W, Coudijzer K, De Vos P, Dewettinck K, Herman L, De Block J, Heyndrickx M. 2009a. Seasonal influence on heat-resistant

proteolytic capacity of *Pseudomonas lundensis* and *Pseudomonas fragi*, predominant milk spoilers isolated from Belgian raw milk samples. Environmental Microbiology 11(2):467-82.

Marchand S, Vandriesche G, Coorevits A, Coudijzer K, De Jonghe V, Dewettinck K, De Vos P, Devreese B, Heyndrickx M, De Block J. 2009b. Heterogeneity of heat-resistant proteases from milk *Pseudomonas* species. International Journal of Food Microbiology 133(1-2):68-77.

Martins ML, Pinto CLO, Rocha RB, de Araújo EF, Vanetti MCD. 2006. Genetic diversity of Gram-negative, proteolytic, psychrotrophic bacteria isolated from refrigerated raw milk. International Journal of Food Microbiology 111(2):144-8.

Murphy S, Boor K. 2000. Trouble-shooting sources and causes of high bacteria counts in raw milk. Dairy, Food and Environmental Sanitation 20(8):606-11.

Nörnberg MFBL, Friedrich RSC, Weiss RDN, Tondo EC, Brandelli A. 2010. Proteolytic activity among psychrotrophic bacteria isolated from refrigerated raw milk. International Journal of Dairy Technology 63(1):41-6.

Perin LM, Moraes PM, Almeida MV, Nero LA. 2012. Intereference of storage temperatures in the development of mesophilic, psychrotrophic, lipolytic and proteolytic microbiota of raw milk. Ciência e Tecnologia de Alimentos 33(1):333-42.

Raats D, Offek M, Minz D, Halpern M. 2011. Molecular analysis of bacterial communities in raw cow milk and the impact of refrigeration on its structure and dynamics. Food Microbiology 28(3):465-71.

Rasolofo EA, St-Gelais D, LaPointe G, Roy D. 2010. Molecular analysis of bacterial population structure and dynamics during cold storage of untreated and treated milk. International Journal of Food Microbiology 138(1-2):108-18.

Regulation. 2004. Regulation (EC) No. 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. Official Journal of the European Union. p. 22-82.

SAS Institute. 2003. The SAS System for Windows version 9.1.3. Cary: SAS Institute Inc.

Scarpellini M, Franzetti L, Galli A. 2004. Development of PCR assay to identify *Pseudomonas fluorescens* and its biotype. FEMS Microbiology Letters 236(2):257-60.

Sørhaug T, Stepaniak L. 1997. Psychrotrophs and their enzymes in milk and dairy products: Quality aspects. Trends in Food Science & Technology 8(2):35-41.

Teh KH, Flint S, Palmer J, Andrewes P, Bremer P, Lindsay D. 2012. Proteolysis produced within biofilms of bacterial isolates from raw milk tankers. International Journal of Food Microbiology 157(1):28-34.

Teh KH, Flint S, Palmer J, Andrewes P, Bremer P, Lindsay D. 2014. Biofilm − An unrecognised source of spoilage enzymes in dairy products? International Dairy Journal 34(1):32-40.

Teh KH, Flint S, Palmer J, Lindsay D, Andrewes P, Bremer P. 2011. Thermo-resistant enzyme-producing bacteria isolated from the internal surfaces of raw milk tankers. International Dairy Journal 21(10):742-7.

Topçu A, Numanoğlu E, Saldamlı İ. 2006. Proteolysis and storage stability of UHT milk produced in Turkey. International Dairy Journal 16(6):633-8.

Tremonte P, Tipaldi L, Succi M, Pannella G, Falasca L, Capilongo V, Coppola R, Sorrentino E. 2014. Raw milk from vending machines: Effects of boiling, microwave treatment, and refrigeration on microbiological quality. Journal of Dairy Science 97(6):3314-20.

Valero E, Villamiel M, Miralles B, Sanz J, Martínez-Castro I. 2001. Changes in flavour and volatile components during storage of whole and skimmed UHT milk. Food Chemistry 72(1):51-8.

Vylětelová M, Hanuš O, Urbanová E. 1999. Occurrence and identification of proteolytic and lipolytic psychrotrophic bacteria in bulk samples of cow's milk. Czech Journal of Food Science 78(9):M1405-M11.

Wiking L, Frøst MB, Larsen LB, Nielsen JH. 2002. Effects of storage conditions on lipolysis, proteolysis and sensory attributes in high quality raw milk. Milchwissenschaft 57(4):190-4.

CHAPTER 2 – Identification and characterization of a heat-resistant protease from *Serratia liquefaciens* isolated from cold raw milk

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Abstract: The cold storage of raw milk before heat treatment in dairy industry promotes the growth of psychrotrophic microorganisms, which are known for their ability to produce heat-resistant proteolytic enzymes. Although *Pseudomonas* is described as the main causative genus for high proteolytic spoilage potential in dairy products, *Serratia liquefaciens* secretes proteases and may be found in raw milk samples as well. However, at the present there is no information about the proteolytic spoilage potential of *S. liquefaciens* in milk after heat-treatment. The main aim of this research was to assess the proteolytic spoilage potential of *S. liquefaciens* isolated from Brazilian raw milk and to characterize the involved protease. *S. liquefaciens* was shown to secrete one metalloprotease of, approximately, 52 kDa. These protease was resistant to 95 °C for 8.45 min and encoded by the *ser2* gene. The heat-resistance of Ser2 was similar to the *aprX* encoded metalloprotease produced by *Pseudomonas*. Although the *ser2* gene was detected in all *S. liquefaciens* isolates tested in this study, the proteolytic activity of the isolates in milk was highly heterogeneous. Since nucleotide and deduced amino acid sequences of *ser2* of all tested isolates are identical, this heterogeneity may be attributed

to differences in enzyme expression levels, post-transcriptional modifications or the presence of other non-identified proteases.

Keywords: metalloprotease, psychrotrophic, *ser2* gene, spoilage.

Highlights

• *Serratia liquefaciens* cause proteolytic spoilage of heat-treated milk similar as *Pseudomonas*.

• Metalloprotease produced by *S. liquefaciens* is highly heat-resistant.

• Heat-resistant metalloprotease is encoded by *ser2* gene in *S. liquefaciens*.

• Spoilage potential of *S. liquefaciens* is heterogeneous among different isolates.

2.1. Introduction

The quality of milk and dairy products may be reduced because of the growth of several different types of microorganisms, resulting in an unacceptable contamination or spoilage. The majority of the bacteria present in raw milk are inactivated by heat treatments commonly used in dairy industry such as pasteurization or ultra-high temperature (UHT) treatment. However, these treatments do not destroy heat-resistant enzymes produced by psychrotrophic bacteria before processing, which represent the main spoilage factor in fluid milk storage (Sørhaug and Stepaniak, 1997).

The heat-resistant metalloprotease from *Pseudomonas* spp. is extensively characterized (Dufour et al., 2008; Kim et al., 1997; Kumeta et al., 1999; Liao and McCallus, 1998; Marchand et al., 2009b) since *Pseudomonas* is the predominant spoilage genus isolated from raw milk samples (Ercolini et al., 2009; Marchand et al., 2009a; Raats et al., 2011; Rasolofo et al., 2010). However, *Serratia* spp. with high spoilage potential was isolated, in addition to *Pseudomonas* species, from Brazilian cold raw milk (Arcuri et al., 2008; Nörnberg et al., 2010) and Italian bulk tank milk (Decimo et al., 2014). Moreover, Machado et al. (2015) highlighted *Pseudomonas* spp. and *Serratia liquefaciens,* identified by molecular methods, are the predominant spoilers found in Brazilian cold raw milk. Both genera, *Serratia* and *Pseudomonas,* isolated from internal surfaces of milk tanks, pasteurizer and holding cells were described as biofilm formers and heat-resistant enzymes producers (Cleto et al., 2012; Teh et al., 2011).

Several studies described metalloproteases from *Serratia marcescens* and *Serratia proteamaculans* (Demidyuk et al., 2006; Jayaratne, 1996; Letoffe et al., 1991; Matsumoto

et al., 1984; Nam et al., 2013; Romero et al., 2001; Tao et al., 2007), although only one study performed by Kaibara et al. (2012) reported proteases from *S. liquefaciens*. As described by these authors, *S. liquefaciens* FK01 produces two serralysin-like metalloproteases, Ser1 and Ser2. However, as far as we know, there is no information in the literature about the heat-resistant metalloprotease(s) from *S. liquefaciens,* which is (are) responsible for spoilage of UHT-treated milk.

The goals of this research were: (i) to assess heat-resistant proteolytic spoilage potential of *S. liquefaciens* in milk in comparison with *Pseudomonas,* (ii) to identify the spoilage responsible heat-resistant protease(s) and the encoding gene(s) from *S. liquefaciens* and (iii) to characterize the kinetic parameters of the spoilage protease(s).

2.2. Materials and Methods

2.2.1. Bacterial isolates and growth conditions

Bacterial isolates used in this work are described in Table 1. All isolates were stored at - 80 °C under cryoprotection with 20 % (v/v) of glycerol. Isolates were grown in Brain Heart Infusion (BHI) (Oxoid, Basingstoke, England) overnight at 22 °C.

2.2.2. Assessment of milk spoilage potential of *S. liquefaciens* and *Pseudomonas* spp.

Strains and isolates were grown in BHI at 22 °C overnight and subsequently, 100 µL of each culture was inoculated in 10 mL semi-skimmed UHT milk and incubated at 22 °C for 24 h. After adaptation in milk, bacterial count on Plate Count Agar (PCA) showed that bacterial population was approximately 10^8 CFU mL^{-1}. All UHT-milk-grown isolates were serially diluted and inoculated in duplicate in 10 mL of fresh UHT milk in a final concentration of 10^4 CFU mL^{-1}. The 10 mL fresh semi-skimmed UHT milk represented the negative control. Milk samples were incubated at three different conditions: at 7 °C for 2 days, at 7 °C for 4 days and at 5 °C for 5 days. The first condition (7 °C for 2 days) is considered adequate for cooling of milk samples in farms according to Brazilian law (Brasil, 2011). The other two conditions are considered as possible extremes. For each condition, a blank (non-inoculated) milk sample was included.

Table 1 - *Serratia* and *Pseudomonas* isolates used in this study.

Name	**Source**
S. liquefaciens DSM 30066	Deutsche Sammlung von Mikroorganismem
S. liquefaciens ATCC 25642 *S. liquefaciens* ATCC 27592T *S. liquefaciens* ATCC 51814	American Type Culture Collection (ATCC)
S. liquefaciens LMG 26065 *S. liquefaciens* LMG 26066	Belgian Co-ordinated Collections of Micro-organisms
S. liquefaciens L53 *S. liquefaciens* L61 *S. liquefaciens* L64 *S. liquefaciens* L79 *S. liquefaciens* L95 *S. liquefaciens* L98 *S. liquefaciens* L104 *S. liquefaciens* L113 *S. liquefaciens* L128 *S. liquefaciens* L130 *S. liquefaciens* L132 *S. liquefaciens* L135 *S. liquefaciens* L136 *S. liquefaciens* L137 *S. liquefaciens* L140 *S. liquefaciens* L146 *S. liquefaciens* L153	Isolates from Brazilian refrigerated raw cow milk[a]
P. fluorescens W2a *P. fragi* Z41b	Isolates from Belgian refrigerated raw cow milk (Marchand et al., 2009a)
P. fluorescens L40 *P. fluorescens* L145 *P. fluorescens* L192 *P. fluorescens* L227 *P. lundensis* L216 *P. lundensis* L231	Isolates from Brazilian refrigerated raw cow milk[a]

[a] *S. liquefaciens*, *P. fluorescens* and *P. lundensis* were the dominant psychrotrophic proteolytic species present in Brazilian raw milk samples stored at simulated cold conditions for farm storage and transport allowed by Brazilian laws (Machado et al., 2015).

After cold storage, milk samples were heated at 95 °C for 8.45 min as described by Marchand et al. (2008) to inactivate plasmin and to discriminate for samples that produce heat stable proteases; subsequently the samples were cooled in ice water. For each isolate, one tube was then incubated at 37 °C for 14 days and a duplicate was stored at -20 °C. Protein hydrolysis was measured by the trinitrobenzenesulfonic acid (TNBS)

method (Marchand et al., 2008; Polychroniadou, 1988) by which the amount of proteolysis is expressed (Eq. 1) as µmol glycine equivalents mL^{-1} using glycine as a standard curve. Increase of protein hydrolysis as a function of time was used to monitor proteolytic activity. The repeatability of the experiment was assessed and confirmed for six isolates (Supplementary Information – Table S1).

(Eq. 1)

$$\frac{\mu\text{mol glycine equivalent}}{\text{mL milk sample}} = (\mu\text{mol glycine equivalent of inoculated milk}_{37°C} - \mu\text{mol glycine equivalent of inoculated milk}_{-20°C}) - (\mu\text{mol glycine equivalent of blank}_{37°C} - \mu\text{mol glycine equivalent of blank}_{-20°C})$$

2.2.3. Protein analysis by electrophoresis

Milk samples were evaluated by zymography to visualize on gel the heat-resistant protease produced by *S. liquefaciens* and to estimate its size. As the heat-resistant protease from *Pseudomonas* spp. is well known and described (Dufour et al., 2008; Marchand et al., 2009b), two isolates of *P. fluorescens* were included as positive controls.

S. liquefaciens and *P. fluorescens* isolates were inoculated in 10 mL of semi-skimmed UHT milk and incubated for 8 days at 7 °C. After incubation, milk samples were heated at 95 °C for 8.45 min (Marchand et al., 2008). After heating, milk samples were cooled in ice water.

Samples were analyzed by zymography as described by Dufour et al. (2008) with some modifications. Fifty microliters of inoculated milk samples were diluted in 1550 µL Zymogram Sample buffer (Bio-Rad, Hercules, CA, USA). An aliquot of 40 µL of diluted sample was loaded in a 12 % Ready Gel® Tris-HCl Gel (Bio-Rad, Hercules, CA, USA) and electrophoresis was performed at room temperature for 1 h under 150 V and 200 mA. After migration, the gel was submerged for 30 min in a solution containing 2 % (w/v) sodium caseinate dissolved in Tris-HCl buffer (0.05 M, pH 7.5) at 7 °C to allow entry of casein. Next, the gel in caseinate solution was incubated at 90 min at room temperature to allow hydrolysis of casein by proteases. After incubation, the gel was stained for 2 h in a solution containing 0.1 % (w/v) Coomassie Brilliant Blue R-250 (Sigma Aldrich, St Louis, MO, USA), 2 % (w/v) trichloroacetic acid (TCA) (Merck, Darmstadt, Germany),

and 50 % (v/v) ethanol (VWR, Leuven, Belgium). Clear zones present after staining correspond to the presence of proteolytic enzymes.

2.2.4. Protein identification by mass spectrometry

To identify the heat-resistant protease produced by *S. liquefaciens*, which showed clear bands on a casein zymogram, the same milk samples that were analyzed by zymography (Section 2.3) were in parallel treated and loaded on a gel under denaturant conditions (SDS-PAGE). The first step of milk samples treatment before loading consisted of casein precipitation by acidification adding HCl 1 M until pH 4.6. Subsequently, samples were centrifuged at 6500 *g* for 5 min. To 1 mL of supernatant 250 µL TCA (100 % w/v) was added, and the tube was centrifuged at 18000 *g* for 5 min to precipitate proteins. The pellet was washed successively with 1 mL ethanol, 1mL ethanol:diethylether (1:1) and 1 mL diethylether. After washing, the pellet was dried for 30 min at room temperature and resuspended in 200 µL Tris-HCl buffer (0.05 M, pH 7.5). Finally, protein concentration was determined by spectrophotometry as described by Warburg and Christian (1942). Measurements were performed using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). Protein solution was diluted in Laemmli Sample Buffer (1:1) (Bio-Rad, Hercules, CA, USA) and 20 µg of protein was loaded on a 12 % Ready Gel® Tris-HCl Gel (Bio-Rad, Hercules, CA, USA). After electrophoresis, proteins were fixed in the gel with a solution of 40 % (v/v) ethanol and 10 % (v/v) acetic acid for 1 h. Next, the gel was stained for 30 min in a solution containing 0.1 % (w/v) Coomassie Blue R250 and 40 % (v/v) ethanol. Finally, the gel was submerged in destaining solution (40 % (v/v) ethanol, 10 % (v/v) acetic acid, 10 % (v/v) glycerol) until the background was clear.

Bands of interest (as determined by zymography) obtained under denaturing conditions were excised from the gel and proteins were identified by mass spectrometry as described by (Marchand et al., 2009b). The peptide mass fingerprint detected by mass spectrometry was examined to verify if it matched with the pattern of trypsin fragmentation known for *Pseudomonas* and *Serratia* proteases. MS/MS was performed on matching peptides to verify their sequence. The protease sequence was compared to NCBI database to identify the encoded gene.

2.2.5. Sequencing of protease gene from *S. liquefaciens*

The putative gene *ser2* (accession number: AB638721.1; expected size: 1461 bp), from *S. liquefaciens*, which encodes a protease was sequenced (Kaibara et al., 2012). All *S. liquefaciens* isolates presented in (Table 1) were grown for 24 h at 22 °C in 10 mL BHI. Subsequently, 1 mL of culture were used for DNA extraction using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). Three pairs of primers were designed based on *S. liquefaciens* ATCC 27592 complete genome (accession number: CP006252.1) to amplify completely *ser2* gene (Table 2).

Table 2 – Primers used to amplify *ser2* gene from *S. liquefaciens* isolates.

Primer	Sequence (5'-3')	Target gene position 5'-3'[a]	Expected PCR product size (bp)
SM1F	AGAGGTGGCATCCATCTTGC	2472948 - 2472967	1040
SM1R	AATCGCATAAGCCTGACCGT	2473987 - 2473978	
SM2F	ATAGCGCGCAGTGAACAAAC	2473700 - 2473719	931
SM2R	TGACATCATTCCCCGAACCG	2474630 - 2474611	
SM3F	GATTTCTACACCGCGACGGA	2474414 - 2474433	982
SM3R	AAGGCGGAATGCCCGATAAT	2475395 - 2475376	

[a] Primers were designed primers based on *S. liquefaciens* ATCC 27592 complete genome (accession number: CP006252.1).

The amplification was carried out in 50 μL reaction mixture containing 20 ng genomic DNA, 0.4 μM of each primer belonging to one pair (SM1F/SM1R or SM2F/SM2R or SM3F/SM3R) (Table 2), 200 μM of each dNTP (Promega, Madison, WI, USA), 2.0 mM $MgSO_4$ (Invitrogen, Carlsbad, NM, USA) and 1 U Platinum® DNA polymerase High Fidelity in 1× High Fidelity PCR buffer (Invitrogen, Waltham, MA, USA). The reaction mixture was denatured at 94 °C for 4 min, followed by 30 cycles (denaturing at 94 °C for 30 s, annealing at 53 °C for 30 s and extension at 68 °C for 1min) and a final elongation step at 68 °C for 10 min in A200 Gradient Thermo Cycler (LongGene, Hangzhou, China). PCR products were checked on agarose gel and sequenced by Macrogen (Seoul, South Korea).

2.2.6. Kinetic parameters for thermal inactivation in semi-skimmed milk of proteases from *S. liquefaciens* and *Pseudomonas* sp.

S. liquefaciens and *Pseudomonas* sp. were inoculated separately in 500 mL of UHT semi-skimmed milk in a final concentration of 10^4 CFU mL^{-1} approximately. After incubation at 7 °C for 65 h about 10^7 CFU mL^{-1} was reached. Milk samples were divided

in aliquots of 5 mL in glass tubes. Samples were heated in a water bath for a certain time interval for each temperature (at 75 °C for 0-420 min; at 85 °C for 0-240min; at 95 °C for 0-150 min). Time necessary to heat up milk (192, 203 and 218 s for heating at 75, 85 and 95 °C, respectively) was determined using TrackSense® Pro Basic Logger (Ellab A/S, Hilleroed, Denmark). Heating time was recorded after milk samples reached the right temperature. After heating, samples were cooled immediately in ice water. Next, 0.025 % bronopol (Merck, Schushardt, Germany) and 0.01 % sodium azide (Merck, Schushardt, Germany) were added to prevent bacterial growth. Subsequently, tubes were incubated at 37 °C for 2 weeks. For each sample, a duplicate was stored at – 20 °C as a blank control to calculate net proteolysis during 2 weeks of storage. Protein hydrolysis was measured by the TNBS method (Marchand et al., 2008). Logarithm of protein hydrolysis level expressed by log (μmol glycine equivalent mL^{-1} milk) was plotted against heating time excluding outliers. The rate constant *k* for first-order inactivation, activation energy (E_a), *D*- and *z*-value were calculated according to Anthon and Barrett (2002).

2.3. Results

2.3.1. Milk spoilage potential of *Pseudomonas* spp. and *S. liquefaciens* under different cold storage conditions

To compare the proteolytic spoilage potential, all 25 isolates from refrigerated raw milk together with reference strains, *S. liquefaciens* ATCC 27592 and LMG 26066 (Table 1) were inoculated in milk samples in a concentration of 10^4 CFU mL^{-1}, which corresponds to the psychrotrophic proteolytic bacterial concentration commonly found in Brazilian raw milk immediately after pouring milk in the cooling tank. Inoculated milk samples were stored under three different conditions.

When milk samples were incubated at 7 °C for 2 days, all isolates reached a concentration between 10^6-10^7 CFU mL^{-1} and 47.8 % of Brazilian isolates, belonging to *P. fluorescens, P. lundensis* and *S. liquefaciens,* presented protein degradation values higher than the threshold value of 0.200 μmol glycine equivalent mL^{-1}. When milk samples were stored at 7 °C for 4 days or 5 °C for 5 days, these Brazilian isolates reached a final concentration of, approximately, 10^8 CFU mL^{-1} and 95.6 % and 85.6 % of Brazilian *S. liquefaciens* and *Pseudomonas* spp. isolates showed levels of proteolysis of higher than the threshould value of 0.200 μmol glycine equivalent mL^{-1}, respectively (Table 3).

The range of proteolysis was 0.000-0.514, 0.147-28.685 and 0.043-27.159 μmol glycine equivalent mL^{-1} considering all tested isolates when samples were incubated at 7 °C for 2 days, 7 °C for 4 days and 5 °C for 5 days respectively. Heterogeneity of proteolytic activity was detected in both genera. The highest proteolytic activity was recorded for *P. fluorescens* of which two isolates (L227, a Brazilian isolate and W2a, a Belgian isolate) showed levels of >20,000 μmol glycine equivalent mL^{-1} after storage at 7 °C for 4 days.

Table 3 – Protein degradation by heat-resistant proteases produced by *S. liquefaciens* and *Pseudomonas* spp. after incubation in milk at three different storage conditions.

Species	Isolates	Storage Conditions					
		7 °C/ 2 days		7 °C/ 4 days		5 °C/ 5 days	
		Log (CFU mL^{-1})	Proteolysis[a]	Log (CFU mL^{-1})	Proteolysis[a]	Log (CFU mL^{-1})	Proteolysis[a]
S. liquefaciens	L53	6.93	0.380	8.3	9.455	8.4	5.337
	L61	6.91	0.194	8.5	5.916	8.3	3.684
	L64	6.73	0.083	8.4	0.338	8.3	0.139
	L79	6.85	0.220	8.4	0.430	8.3	0.272
	L95	6.78	0.000	8.4	2.395	8.3	1.919
	L98	6.99	0.320	8.5	4.393	8.3	5.602
	L104	6.14	0.000	7.9	0.297	8.0	0.350
	L113	6.64	0.106	8.3	0.365	8.3	0.224
	L128	6.58	0.143	8.2	0.305	8.2	0.300
	L130	6.54	0.051	8.2	1.475	8.0	0.884
	L132	7.03	0.371	8.4	6.185	8.3	3.866
	L135	6.85	0.514	8.3	4.215	8.3	3.080
	L136	6.74	0.042	8.5	0.273	8.2	0.095
	L137	6.56	0.277	8.2	0.877	8.0	0.360
	L140	6.88	0.360	8.4	4.194	8.2	2.770
	L146	6.82	0.382	8.4	4.639	8.2	2.605
	L153	6.30	0.053	8.0	0.578	7.7	0.198
	ATCC 27592	6.64	0.000	8.2	0.454	8.1	0.336
	LMG 26066	6.41	0.162	8.2	0.258	8.1	0.371
P. fluorescens	L40	6.40	0.000	7.8	0.481	7.7	0.267
	L145	6.77	0.000	8.3	2.652	8.0	3.502
	L192	6.73	0.265	8.4	3.117	8.2	1.985
	L227	6.60	0.265	8.0	28.685	8.0	27.159
	W2a	6.65	0.000	7.8	20.153	8.0	12.312
P. fragi	Z41b	7.15	0.000	8.3	10.693	8.2	12.325
P. lundensis	L231	7.10	0.465	7.8	0.147	7.7	0.043
	L216	7.04	0.000	7.8	0.153	7.9	0.226

[a] Proteolysis was expressed in μmol glycine equivalents mL^{-1}.

2.3.2. Analysis of protease by casein zymography

S. liquefaciens L53, L79, L98, L104 and *P. fluorescens* L227 and W2a were incubated in semi-skimmed UHT milk for 8 days at 7 °C and their heat-resistant proteolytic activity was assessed by casein zymography.

Clear zones detected in zymogram correspond to proteolytic activity. Only one band of protease from *S. liquefaciens* L53, L98, L104 and *P. fluorescens* L227 and W2a was visualized by zymography (Figure 1). However, the protease detected from *S. liquefaciens* was larger with, approximately, 52 kDa of apparent molecular weight. Moreover, *S. liquefaciens* L79 was the only tested isolate that did not exhibit extracellular activity in zymogram (Figure 1).

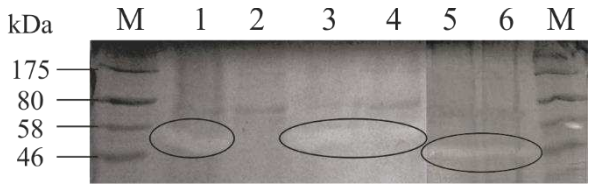

Figure 1 – Detection of proteases by casein zymography under non-reducing conditions. Clear zones indicate casein hydrolysis (circles). The isolates *S. liquefaciens* L53 (1), L79 (2), L98 (3), L104 (4) and *P. fluorescens* L227 (5) and W2a (6) were inoculated in UHT milk incubated for 8 days at 7 °C.

2.3.3. Confirmation of protease identity by mass spectrometry

The bands from *S. liquefaciens* L53 and L79 visualized on SDS-PAGE corresponding to proteolytic activity detected by zymography (L53) or on the same height (L79) were analyzed by mass spectrometry to identify this protease. MS/MS spectra of selected peptides from the peptide mass fingerprint were searched using the Mascot algorithm (www.matrixscience.com) against the NCBI database and matched best to serralysin-like metalloprotease 2. Five peptides retrieved (shown in bold in Figure 2) by mass spectrometry analysis corresponded perfectly to the deduced amino acid sequences of a protein from *S. liquefaciens* ATCC27592 annotated as a serine 3-dehydrogenase in NCBI and *ser2* gene of *S. liquefaciens* Kuo1-1. The protein from *S. liquefaciens* ATCC27592 annotated as a serine 3-dehydrogenase presents the Zn^{+2} and Ca^{+2} binding motif, which demonstrate this protein is a metalloprotease. Thus, this serine 3-dehydrogenase in NCBI should be reannotated.

The active heat-resistant protease from *S. liquefaciens* isolates from Brazilian cold raw milk is a product of *ser2* gene, which encodes a heat-stable protease with molecular weight of, approximately, 52 kDa in *S. liquefaciens* isolates.

2.3.4. Sequencing of *ser2* gene of *S. liquefaciens*

The pairs of primers SM1F/SM1R, SM2F/SM2R and SM3F/SM3R (Table 2) were used to amplify the gene *ser2* of *S. liquefaciens* isolates, and its upstream and downstream regions. The sequences of three overlapping PCR products allowed to obtain a sequence between 2308 and 2396 bp for 23 *S. liquefaciens* isolates. It contained an open reading frame (ORF) of 487 amino acids (1461 bp) and shared 94 % identity on nucleotide level with the *ser2* gene of *S. liquefaciens* Kuo1-1 (accession number: BAK39733; Egami et al. (2009)) and 99 % identity with the region encoding a metalloprotease in the complete genome (position 5'-3': 2473557-2475029) of *S. liquefaciens* ATCC 27592 (accession number: CP006252.1).

The deduced amino acid sequences of Ser2 from all 23 *S. liquefaciens* isolates matched with Ser2 of *S. liquefaciens* ATCC27592 and Kuo1-1. Even Ser2 sequence from *S. liquefaciens* L79, which did not show proteolytic activity by zymography, matched 100 % with *S. liquefaciens* L53, which is the most proteolytic isolate among *S. liquefaciens* isolates tested in this study (Figure 2).

Ser2 is a metalloprotease that belongs to the peptidase family M10 (Rawlings et al., 2013). Proteases from family M10 present the Zn^{+2} binding motif HEXXHXXGXXH in which Glu is presumed to be the catalytic residue. Furthermore, heat resistant protease from *S. liquefaciens* has multiple tandem repeats of a nine residue sequence which includes a GGXGXD consensus motif (in which X is an arbitrary amino acid) and which binds Ca^{2+} ions (Baumann et al., 1993) and a ABC exported motif (DXXX). These motifs could be detected in protein sequences deduced from the *ser2* gene of Brazilian milk isolates (Figure 2).

```
S. liquefaciens ATCC 27592 MDNSLNGKNNGWDSVNDLLNYHNRGNGLTINNKPSFDIAAAGKQIARSEQTWNGTHVLGQ 60
S. liquefaciens L53        MDNSLNGKTNGWDSVNDLLNYHNRGNGLTINNKPSFDIAAAGKQIARSEQTWNGTHVLGQ 60
S. liquefaciens L79        MDNSLNGKTNGWDSVNDLLNYHNRGNGLTINNKPSFDIAAAGKQIARSEQTWNGTHVLGQ 60
                                                    -

S. liquefaciens ATCC 27592 GATVTYSFPDWDYNQSNLNGRFASQDTGLSAFTADQKAQAKLSLQSWADVANLNFVEVA 119
S. liquefaciens L53        GATVTYSFPDWDYNQSNLNGRFASQDTGLSAFTADQKAQAKLSLQSWADVANLNFVEVA 119
S. liquefaciens L79        GATVTYSFPDWDYNQSNLNGRFASQDTGLSAFTADQKAQAKLSLQSWADVANLNFVEVA 119

S. liquefaciens ATCC 27592 PGQKSNITFGNYEGDGQAYAIKPFTGAGTDYRGHNTDGQSWFNINYDYADPRDGVYANL 178
S. liquefaciens L53        PGQKSNITFGNYEGDGQAYAIKPFTGAGTDYRGHNTDGQSWFNINYDYADPRDGVYANL 178
S. liquefaciens L79        PGQKSNITFGNYEGDGQAYAIKPFTGAGTDYRGHNTDGQSWFNINYDYADPRDGVYANL 178

S. liquefaciens ATCC 27592 HPELGNYGRLSITHELGHTLGLDHPGVYNAGQSPSYAKATYAEDTRQFSVMSYWDESVT 237
S. liquefaciens L53        HPELGNYGRLSITHELGHTLGLDHPGVYNAGQSPSYAKATYAEDTRQFSVMSYWDESVT 237
S. liquefaciens L79        HPELGNYGRLSITHELGHTLGLDHPGVYNAGQSPSYAKATYAEDTRQFSVMSYWDESVT 237

S. liquefaciens ATCC 27592 GGDHGGYYSAAPLVDDIAAIQYLYGANTTTRTGDTVYGFNSNSGRDFYTATDSSQKLIF 296
S. liquefaciens L53        GGDHGGYYSAAPLVDDIAAIQYLYGANTTTRTGDTVYGFNSNSGRDFYTATDSSQKLIF 296
S. liquefaciens L79        GGDHGGYYSAAPLVDDIAAIQYLYGANTTTRTGDTVYGFNSNSGRDFYTATDSSQKLIF 296

S. liquefaciens ATCC 27592 SVWDAGGNDTLDFSGYSQDQRINLTEGSFSDVGGLKGNISIAVGAVIENAIGGSGNDVI 355
S. liquefaciens L53        SVWDAGGNDTLDFSGYSQDQRINLTEGSFSDVGGLKGNISIAVGAVIENAIGGSGNDVI 355
S. liquefaciens L79        SVWDAGGNDTLDFSGYSQDQRINLTEGSFSDVGGLKGNISIAVGAVIENAIGGSGNDVI 355

S. liquefaciens ATCC 27592 VGNDAANILQGGAGNDVIYGGGGQDQLSGGSGSDIFVFSAVSDSPFKSPDKILDFETGI 414
S. liquefaciens L53        VGNDAANILQGGAGNDVIYGGGGQDQLSGGSGSDIFVFSAVSDSPFKSPDKILDFETGI 414
S. liquefaciens L79        VGNDAANILQGGAGNDVIYGGGGQDQLSGGSGSDIFVFSAVSDSPFKSPDKILDFETGI 414

S. liquefaciens ATCC 27592 DKIDLSFFNQGDNGTDFIHFVDSFSGQAGEATLTYNSQSDFSELALNISGHATPDFLVN 473
S. liquefaciens L53        DKIDLSFFNQGDNGTDFIHFVDSFSGQAGEATLTYNSQSDFSELALNISGHATPDFLVN 473
S. liquefaciens L79        DKIDLSFFNQGDNGTDFIHFVDSFSGQAGEATLTYNSQSDFSELALNISGHATPDFLVN 473

S. liquefaciens ATCC 27592 IVGQANTATDFIV 486
S. liquefaciens L53        IVGQANTATDFIV 486
S. liquefaciens L79        IVGQANTATDFIV 486
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Figure 2 – Sequence alignment of deduced amino acid sequences of complete *ser2* gene obtained for *S. liquefaciens* ATCC 27592 (reference strain, Accession Number: AGQ31112), *S. liquefaciens* L53 and L79 isolated from Brazilian cold raw milk. Peptides as retrieved by mass spectrometry are shown in bold. The underlined peptides represent Zn^{+2} binding motif, Ca^{+2} binding motif and ABC exporter motif. Symbol (-) represents mismatching.

2.3.5. Kinetic parameters for thermal inactivation of proteases from *S. liquefaciens* and *Pseudomonas* sp. in semi-skimmed milk

The most proteolytic isolate belonging to *S. liquefaciens* (L53) and the most proteolytic isolate well characterized by Marchand et al. (2009b) belonging to *P. fluorescens* (W2a) were inoculated in semi-skimmed UHT milk. After incubation at 7 °C,

samples were heated to evaluate kinetic parameters for thermal inactivation of Ser2 and to compare with AprX.

The extent of proteases denaturation increased with temperature and heating time. Thermal inactivation of proteases from *S. liquefaciens* L53 and *P. fluorescens* W2a were linear at three evaluated temperatures (Figure 3). R^2-values of all inactivation curves are presented in Table 4. From the slopes, inactivation rate constants (k) were calculated (Table 4). Inactivation rate constants increased with heating temperature, indicating that proteases from these psychrotrophic bacteria were less stable at higher temperatures. Half-life ($t_{1/2}$) and D-values decreased with temperature increase (Table 4).

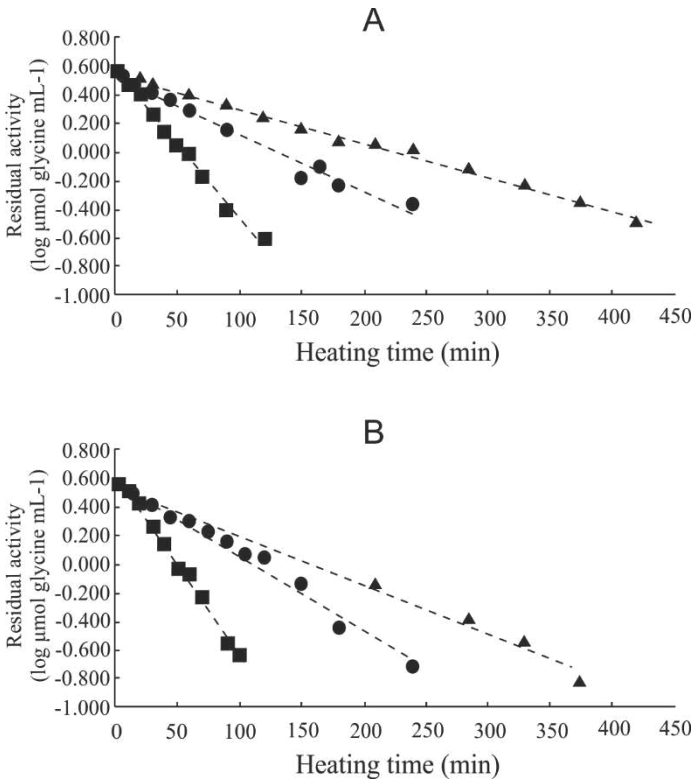

Figure 3 – Inactivation of heat-resistant proteolytic activity from *S. liquefaciens* L53 (A) and *P. fluorescens* W2a (B) in milk heated at 75 °C (triangles), 85 °C (circles) and 95 °C (squares). Residual proteolytic activity is represented by log (µmol glycine equivalents mL^{-1}) as a function of heating time.

Table 4 – Calculated E_a, D-, k-, $t_{1/2}$-, R^2- and z-values for heat-resistant proteolytic activity from *S. liquefaciens* L53 and *P. fluorescens* W2a.

		Temperature (°C)		
		75	**85**	**95**
S. liquefaciens	*D* (min)	416.67	250.00	96.15
	k (min^{-1})	0.006	0.009	0.024
	$t_{1/2}$	125.41	75.24	28.94
	R^2	0.9954	0.9817	0.995
	E_a (kJ mol^{-1})		77.89	
	z-value (°C)		31.45	
Pseudomonas sp.	*D* (min)	294.12	188.68	80.00
	k (min^{-1})	0.008	0.012	0.029
	$t_{1/2}$	88.52	56.79	24.08
	R^2	0.987	0.9823	0.9923
	E_a (kJ mol^{-1})		69.15	
	z-value (°C)		35.34	

The dependence of the inactivation rate constants with temperature was adequately fitted by the Arrhenius equation (not shown). Based on Arrhenius plot, the activation energy (E_a) was calculated. The low E_a and high *z*-values (Table 4) confirm that proteases from *S. liquefaciens* and *Pseudomonas* sp. are heat-resistant.

2.4. Discussion

The proteolysis of casein may cause destabilization of milk with formation of gel or sediments. Milk gelation and sedimentation is related to poor quality of milk. Although identifying the causes of milk destabilization is a challenge, it is well known that the presence of microorganisms as producers of heat-resistant protease in raw milk may be a possible cause. *Pseudomonas* spp. was described as the main genus related to casein proteolysis, but recent studies highlighted that *Serratia* spp., which may secrete protease, were detected in cold raw milk samples (Cleto et al., 2012; Decimo et al., 2014; Machado et al., 2015). However, there is no information about the potential of *Serratia* spp. in spoilage of heat-treated milk and which of the described proteases (Ser1 and Ser2) in this genus is responsible for heat resistant proteolytic activity in milk.

The ability of *Serratia* spp. to produce a protease has been evaluated by a plate assay using media containing milk. After incubation, proteolysis was monitored by

detection of clear haloes (Cleto et al., 2012; Decimo et al., 2014). However, this simple screening assay does not give an indication of heat-resistance of this proteolytic activity (Bauer et al., 2015). These authors highlighted that false positive results for proteolytic activity on plate assay can arise from acid formation from lactose by glycoside hydrolases in clear hydrolysis zones on the agar plates.

Besides the plate assay, the proteolytic activity of *Pseudomonas* and *S. liquefaciens* cultures was quantified by TNBS method. Our study revealed that *S. liquefaciens* may produce heat-resistant proteolytic activity similar to *Pseudomonas* spp., which can contribute to reduced shelf life of heat-treated milk. When these proteolytic psychrotolerant bacteria were grown in milk samples at 7 °C for 2 days, proteolysis was much lower compared to more extreme but still possible storage conditions. These results evidenced that raw milk must be stored in adequate conditions (e.g. <7 °C for 2 days) to prevent the production of heat-resistant proteases, which may lead to the reduction of dairy products shelf life. Furthermore, this study confirmed that the bacteriological and enzymatic quality of cold raw milk is highly influenced by storage conditions.

Casein zymography in association with mass spectrometry evidenced that *S. liquefaciens* produces a heat-resistant protease with an apparent molecular weight of about 52 kDa, which is different from *Pseudomonas* spp., which secrete a heat-resistant protease of 45 kDa (Dufour et al., 2008; Marchand et al., 2009b). This finding was reinforced by the study performed by Decimo et al. (2014) in which the *aprX* gene that encodes the heat-resistant metalloprotease in *Pseudomonas* spp. was not found in the *Serratia* genome. It was found that the heat-resistant proteases secreted by *S. liquefaciens* and *Pseudomonas* spp. are encoded by different genes, *ser2* and *aprX* respectively, as well as that the other protease of *S. liquefaciens*, encoded by *ser*1, was apparently not related to the heat-resistant proteolytic activity. No clear band corresponding to proteolytic activity was visualized when protein extract from *S. liquefaciens* L79 was analyzed by zymography. This corresponds with the low proteolytic activity (0.430 µmol glycine equivalent/mL) detected by TNBS method from isolate L79 after 4 days incubation at 7 °C. Probably, this isolate produces heat-resistant protease in a concentration lower than could be detected by zymography. The expressive variation in proteolysis (0.258 - 9.455 µmol glycine equivalents mL^{-1}) of milk samples inoculated with different *S. liquefaciens* isolates (Table 3) indicated heterogeneity in proteolytic

potential among milk isolates. The heterogeneity of proteolytic activity in *Pseudomonas* spp. was also highlighted by Dufour et al. (2008) and Marchand et al. (2009b).

The genomic DNA from 23 *S. liquefaciens* isolates was used as template to amplify and sequence the *ser2* gene, which encodes the heat-resistant metalloprotease secreted by this species as confirmed after peptide sequence analysis by mass spectrometry. Although no significant similarity was found among *ser1*, *ser2* and *aprX* sequences, the same primers may be used to amplify a partial sequence of *aprX* from *P. fluorescens* and *ser1* from *S. marcescens* (Woods et al., 2001). The nucleotide sequences of *ser2* gene presented 100 % of identity when the 23 isolates were compared, thus the heterogeneity in caseinolytic activity could not be attributed to the difference of *ser2* sequences. This feature of *S. liquefaciens* differs from that of *Pseudomonas* spp. since Marchand et al. (2009b) revealed a large heterogeneity in *aprX* sequences from milk isolates belonging to *Pseudomonas*. The high variability in spoilage potential of *S. liquefaciens* may be explained by heterogeneity in enzyme expression, post-transcriptional modifications or the presence of other non-identified proteases.

A kinetic study of isothermal inactivation of proteases from *P. fluorescens* and *S. liquefaciens* was performed at 75, 85 and 95 °C with different heating times corresponding to heat treatment commonly used in dairy industry such as pasteurization or UHT. Results of thermal inactivation analysis showed *P. fluorescens* and *S. liquefaciens* protease were partially inactivated by means of a simple first-order kinetic process. The fact that the curves at different temperatures extrapolate back to a common point indicates that the inactivation of a single isoenzyme is being measured (Gouzi et al., 2011). Zymography and mass spectrometry analysis confirmed that both species produce a single but different heat-resistant protease still active after heating at 95 °C for 8.45 min when inoculated in milk incubated at 7 °C. The heat resistance of extracellular protease (AprX) from *Pseudomonas* spp. is widely known (Baglinière et al., 2013; Gaucher et al., 2011; Marchand et al., 2009b; Matselis and Roussis, 1998; Mu et al., 2009). However, this is the first study that demonstrated *S. liquefaciens* isolates from raw milk produce a heat stable protease encoded by the *ser2* gene, which may compromise milk products quality. Compared to the *D*-value of heat-resistant proteolytic activity of bacterial origin in Belgian raw milk at 75 °C (*D*-value = 362.77 min) and 85 °C (*D*-value = 200.73) (Marchand et al., 2008), it was shown that the Ser2 protease from *S. liquefaciens* was at least similarly thermostable (D-value = 416.67 min and 250 min, respectively).

2.5. Conclusion

The metalloprotease produced by *S. liquefaciens* and encoded by the *ser2* gene is highly heat-resistant comparable to the AprX metalloprotease from *Pseudomonas* spp., thus *S. liquefaciens*, being a psychrotolerant microorganism, may cause technological problems in the dairy industry. Although, this research has demonstrated that the spoilage potential of *S. liquefaciens* is isolate dependent, no variability concerning nucleotide and deduced amino acid sequences of *ser2* was detected. Further studies should be performed with the purpose of pointing the cause of this heterogeneity in heat-resistant proteolytic activity, which will allow the implementation of more effective measures to trace harmful isolates in order to minimize the proteolysis in dairy products.

This study evidenced that there are more proteases, which can be active after heat treatment, than the proteases described in the literature. Thus, the dairy industry needs a method, which could detect and quantify the total protease activity regardless of the origin of proteases that are in milk samples. The method based on hydrolysis of casein coated in a microplate, which described in the Chapter 3, was developed in an attempt of to become useful for predicting the milk quality in dairy industry.

2.6. References

Anthon, G.E., Barrett, D.M., 2002. Kinetic parameters for the thermal inactivation of quality-related enzymes in carrots and potatoes. Journal of Agricultural and Food Chemistry 50, 4119-4125.

Arcuri, E.F., Silva, P.D.L., Brito, M.A.V.P., Brito, J.R.F., Lange, C.C., Magalhães, M.M.A., 2008. Contagem, isolamento e caracterização de bactérias psicrotróficas contaminantes de leite cru refrigerado. Ciência Rural 38, 2250-2255.

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

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

Baumann, U., Wu, S., Flaherty, K.M., McKay, D.B., 1993. Three-dimensional structure of the alkaline protease of *Pseudomonas aeruginosa*: a two-domain protein with a calcium binding parallel beta roll motif. The EMBO Journal 12, 3357.

Brasil, 2011. Regulamento Técnico de Produção, Identidade e Qualidade do Leite tipo A, Regulamento Técnico de Identidade e Qualidade de Leite Cru Refrigerado, Regulamento Técnico de Identidade e Qualidade de Leite Pasteurizado e Regulamento Técnico da Coleta de Leite Cru Refrigerado e seu Transporte a Granel, Instrução Normativa nº 62, de 29 de de dezembro de 2011. Ministério da Agricultura, Pecuária e Abastecimento, Diário Oficial [da] República Federativa do Brasil, Brasília, DF, pp. 6-37.

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

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

Demidyuk, I.V., Kalashnikov, A.E., Gromova, T.Y., Gasanov, E.V., Safina, D.R., Zabolotskaya, M.V., Rudenskaya, G.N., Kostrov, S.V., 2006. Cloning, sequencing, expression, and characterization of protealysin, a novel neutral proteinase from *Serratia proteamaculans* representing a new group of thermolysin-like proteases with short N-terminal region of precursor. Protein Expression and Purification 47, 551-561.

Dufour, D., Nicodème, M., Perrin, C., Driou, A., Brusseaux, E., Humbert, G., Gaillard, J.-L., Dary, A., 2008. Molecular typing of industrial strains of *Pseudomonas* spp. isolated

from milk and genetical and biochemical characterization of an extracellular protease produced by one of them. International Journal of Food Microbiology 125, 188-196.

Egami, I., Iiyama, K., Zhang, P., Chieda, Y., Ino, N., Hasegawa, K., Lee, J., Kusakabe, T., Yasunaga-Aoki, C., Shimizu, S., 2009. Insecticidal bacterium isolated from an ant lion larva from Munakata, Japan. Journal of Applied Entomology 133, 117-124.

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

Gaucher, I., Tanguy, G., Fauquant, J., Jardin, J., Rousseau, F., Robert, B., Madec, M.-N., Gaucheron, F., 2011. Proteolysis of casein micelles by *Pseudomonas fluorescens* CNRZ 798 contributes to the destabilisation of UHT milk during its storage. Dairy Science & Technology 91, 413-429.

Gouzi, H., Depagne, C., Coradin, T., 2011. Kinetics and thermodynamics of the thermal inactivation of polyphenol oxidase in an aqueous extract from *Agaricus bisporus*. Journal of Agricultural and Food Chemistry 60, 500-506.

Jayaratne, P., 1996. Major metalloprotease gene of *Serratia marcescens* is conserved and provides a molecular typing method to differentiate clinical isolates. Journal of Microbiological Methods 26, 261-269.

Kaibara, F., Iiyama, K., Chieda, Y., Man Lee, J., Kusakabe, T., Yasunaga-Aoki, C., Shimizu, S., 2012. Construction of serralysin-like metalloprotease-deficient mutants of *Serratia liquefaciens* and their virulence in the silkworm, *Bombyx mori*. Journal of Insect Biotechnology and Sericology 81, 55-61.

Kim, H.-J., Tamanoue, Y., Jeohn, G.-H., Iwamatsu, A., Yokota, A., Kim, Y.-T., Takahashi, T., Takahashi, K., 1997. Purification and characterization of an extracellular metalloprotease from *Pseudomonas fluorescens*. Journal of Biochemistry 121, 82-88.

Kumeta, H., Hoshino, T., Goda, T., Okayama, T., Shimada, T., Ohgiya, S., Matsuyama, H., Ishizaki, K., 1999. Identification of a member of the serralysin family isolated from a psychrotrophic bacterium, *Pseudomonas fluorescens* 114. Bioscience Biotechnology Biochemistry 63, 1165-1170.

Letoffe, S., Delepelaire, P., Wandersman, C., 1991. Cloning and expression in *Escherichia coli* of the *Serratia marcescens* metalloprotease gene: secretion of the protease from *E. coli* in the presence of the *Erwinia chrysanthemi* protease secretion functions. Journal of Bacteriology 173, 2160-2166.

Liao, C.-H., McCallus, D.E., 1998. Biochemical and genetic characterization of an extracellular protease from *Pseudomonas fluorescens* CY091. Applied and Environmental Microbiology 64, 914-921.

Machado, S.G., Silva, F.L., Bazzolli, D.M.S., Heyndrickx, M., Costa, P.M.A., Vanetti, M.C.D., 2015. *Pseudomonas* spp. and *Serratia liquefaciens* as predominant spoilers in Brazilian cold raw milk. Journal of Food Science, *In press*.

Marchand, S., Coudijzer, K., Heyndrickx, M., Dewettinck, K., De Block, J., 2008. Selective determination of the heat-resistant proteolytic activity of bacterial origin in raw milk. International Dairy Journal 18, 514-519.

Marchand, S., Heylen, K., Messens, W., Coudijzer, K., De Vos, P., Dewettinck, K., Herman, L., De Block, J., Heyndrickx, M., 2009a. Seasonal influence on heat-resistant proteolytic capacity of *Pseudomonas lundensis* and *Pseudomonas fragi*, predominant milk spoilers isolated from Belgian raw milk samples. Environmental Microbiology 11, 467-482.

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

Matselis, E., Roussis, I.G., 1998. Proteinase and lipase production by *Pseudomonas fluorescens*. Proteolysis and lipolysis in thermized ewe's milk. Food Control 9, 251-259.

Matsumoto, K., Maeda, H., Takata, K., Kamata, R., Okamura, H., 1984. Purification and characterization of four proteases from a clinical isolate of *Serratia marcescens* kums 3958. Journal of Bacteriology 157, 255-232.

Mu, Z., Du, M., Bai, Y., 2009. Purification and properties of a heat-stable enzyme of *Pseudomonas fluorescens* Rm12 from raw milk. European Food Research and Technology 228, 725-734.

Nam, M.S., Whang, K.S., Choi, S.H., Bae, H.C., Kim, Y.K., Park, Y.W., 2013. Purification, characterization, and properties of an alkaline protease produced by *Serratia marcescens* S3-R1 inhabiting Korean ginseng rhizosphere. Journal of the Science of Food and Agriculture 93, 3876-3882.

Nörnberg, M.F.B.L., Friedrich, R.S.C., Weiss, R.D.N., Tondo, E.C., Brandelli, A., 2010. Proteolytic activity among psychrotrophic bacteria isolated from refrigerated raw milk. International Journal of Dairy Technology 63, 41-46.

Polychroniadou, A., 1988. A simple procedure using trinitrobenzenesulphonic acid for monitoring proteolysis in cheese. Journal of Dairy Research 55, 585-596.

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

Rasolofo, E.A., St-Gelais, D., LaPointe, G., Roy, D., 2010. Molecular analysis of bacterial population structure and dynamics during cold storage of untreated and treated milk. International Journal of Food Microbiology 138, 108-118.

Rawlings, N.D., Waller, M., Barrett, A.J., Bateman, A., 2013. MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Research 40, D443-D450.

Romero, F.J., García, L.A., Salas, J.A., Díaz, M., Quirós, L.M., 2001. Production, purification and partial characterization of two extracellular proteases from *Serratia marcescens* grown in whey. Process Biochemistry 36, 507–515.

Sørhaug, T., Stepaniak, L., 1997. Psychrotrophs and their enzymes in milk and dairy products: Quality aspects. Trends in Food Science & Technology 8, 35-41.

Tao, K., Yu, X., Liu, Y., Shi, G., Liu, S., Hou, T., 2007. Cloning, expression, and purification of insecticidal protein Pr596 from locust pathogen *Serratia marcescens* HR-3. Current Microbiology 55, 228-233.

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

Warburg, O., Christian, W., 1942. Isolation and crystallization of enolase. Biochemische Zeitschrift 310, 384-421.

Woods, R.G., Burger, M., Beven, C.-A., Beacham, I.R., 2001. The *aprX-lipA* operon of *Pseudomonas fluorescens* B52: a molecular analysis of metalloprotease and lipase production. Microbiology 147, 345-354.

Table S1 – Assessment of the repeatability of the experiment performed in order to quantify the protein degradation by heat-resistant proteases produced by *S. liquefaciens* and *Pseudomonas* spp. after incubation in milk at two different storage conditions.

		Repetition A		**Repetition B**		**Repetition C**		Log (CFU mL^{-1})		Proteolysis	
Storage Conditions	**Strains**	Log (CFU mL^{-1})	Proteolysis	Log (CFU mL^{-1})	Proteolysis	Log (CFU mL^{-1})	Proteolysis	Average	SD	Average	SD
7 °C for 2 days	L53	7.1	0.512	7.1	0.607	7.2	0.566	7.1	0.1	0.562	0.048
	L79	7.1	0.271	7.0	0.327	7.1	0.243	7.1	0.0	0.280	0.043
	L98	7.0	0.593	7.2	0.611	7.1	0.663	7.1	0.1	0.622	0.036
	L104	6.3	0.056	6.3	0.139	6.3	0.160	6.3	0.0	0.118	0.055
	L227	6.7	0.387	6.5	0.339	7.0	0.345	6.7	0.2	0.357	0.026
	W2a	6.6	0.197	6.7	0.330	6.8	0.350	6.7	0.1	0.292	0.083
7 °C for 4 days	L53	8.6	9.761	8.7	9.182	8.7	9.482	8.7	0.0	9.475	0.290
	L79	8.6	0.034	8.8	0.668	8.5	0.762	8.6	0.1	0.488	0.396
	L98	8.7	7.488	8.8	8.213	8.7	7.846	8.7	0.1	7.849	0.363
	L104	8.2	0.378	8.3	0.382	8.2	0.401	8.2	0.1	0.387	0.012
	L227	8.4	34.160	8.4	32.121	8.4	38.776	8.4	0.0	35.019	3.410
	W2a	8.4	32.005	8.3	33.303	8.2	34.429	8.3	0.1	33.246	1.213

CHAPTER 3 - Development of an assay on solid phase for detecting proteases in milk

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Abstract: Quality of dairy products is closely related to microbial enzymes such as proteases, which are still actives after heat treatment. Since there is no efficient method for measurement of proteolytic activity in milk samples in a short period, dairy industry needs a rapid and accurate assay for assessment of milk quality based on protease detection. A solid-phase assay proteases using biotinylated casein was evaluated for milk analysis. The assay involves coating microtiter plates with biotinylated casein, hydrolysis of this substrate by proteases and quantification of remaining casein. The quantification is based on a reaction between biotin and streptavidin peroxidase conjugate followed by peroxidase activity as an indicator. Trypsin, papain, pepsin, thermolysin and protease from bovine pancreas hydrolyzed the substrate bound on plates to varying degrees. However, proteolytic activity of milk samples containing microbial proteases could not be measured efficiently. This assay is cheap, simple and useful for detecting all proteases found in milk samples even the unknown enzymes, but it has to be improved before being deployed in dairy industry.

Keywords: quantification, proteolytic activity; thermolysin; metalloprotease; biotin; streptavidin.

3.1. Introduction

A major cause of dairy product spoilage is microbial enzymes such as proteases, which may remain active in food after proteolytic microorganisms have been destroyed by heat treatment (Ledenbach and Marshall, 2010). Proteases produced by psychrotrophic bacteria have an important role in dairy products shelf life because these enzymes may resist heat-treatment commonly used in industry as pasteurization and ultra-high temperature processing (UHT). The presence of heat-resistant proteases in milk cause instability problems or defects that are detectable by sensory tests (Sørhaug and Stepaniak, 1997).

To prevent deterioration of dairy products before expiration date, rapid detection of heat-resistant proteases is very convenient. The standard plate count procedure is often employed in milk and dairy products to detect psychrotrophic microorganisms, which may produce heat-resistant enzymes. However, this method is time-consuming and does not allow for the rapid assessment of food spoilage potential (Dufour et al., 2008; Vanetti, 2009). None of the tests commonly used by the fluid milk industry to screen raw milk has the ability to predict the shelf life and sensory quality of heat-treated milk (Martin et al., 2011).

Rapid methods as PCR have been developed to detect specific genes encoding protease in predominant milk spoilers (Machado et al., 2013; Martins et al., 2005). However, Baglinière et al. (2012) e Dufour et al. (2008) proved that some *Pseudomonas* strains did not secrete extracellular metalloprotease although the gene encoding this enzyme has been detected. Therefore, the detection of proteases genes by PCR is not the recommended method to predict milk quality. The dairy industry needs a scientifically valid milk test that will be able to predict quality and shelf life of milk. Thus, the detection of these proteases in milk can be an alternative tool to assess the quality of heat-treated milk products.

Since heat-resistant proteases produced by predominant spoilers are very heterogeneous (Dufour et al., 2008; Marchand et al., 2009b), it would be interesting to create a method that can detect heat-resistant proteases in general. This study proposed an assay on a solid phase based on detection of remained biotinylated

casein coated on microplates after hydrolysis by proteases. The goals of this work were: (i) to optimize parameters that can influence the sensitivity of the developed assay; (ii) to validate the assay for detecting proteases diluted in buffer and (iii) to assess the method for milk samples analyzing.

3.2. Materials and Methods

3.2.1. Reagents and buffers

The developed assay was performed on high binding MICROLON® 600 microplates with 96 well and solid F-bottom (Greiner bio-one GmbH, Frickenhausen, Germany) coated with casein from bovine milk purchased from Sigma-Aldrich (St Louis, MO, USA), which was biotinylated by Ibt – immunological and biochemical testsystems GmbH (Binzwangen, Germany). The biotinylated casein (BC) was diluted in phosphate buffered saline pH 7.4 (PBS) (Sigma Aldrich, St Louis, MO, USA). Thermolysin from *Bacillus thermoproteolyticus rokko* (EC 3.4.24.27), pepsin A (EC 3.4.23.1), trypsin from bovine pancreas (3.4.21.4), papain Carica Papaya (EC 3.4.22.2), protease from bovine pancreas, 3,3',5,5'-tetramethylbenzidine (TMB), dimethyl sulfoxide (DMSO), hydrogen peroxide (30 %) and Tween 20 were purchased from Sigma-Aldrich (St Louis, MO, USA). Protein-free T20 blocking buffer, Streptavidin Horseradish Peroxidase Conjugated (streptavidin-HRP) and High Sensitivity Streptavidin-HRP were purchased from Pierce Biotechnology (Rockford, USA). Bovine Serum Albumin (BSA) and SmartBlockTM were acquired from Roche Diagnostics Belgium N.V. (Vilvoorde, Belgium) and CANDOR Bioscience GmbH (Wangen, Germany) respectively.

Phosphate buffer (pH 6.5) consisted of 0.15 M NaCl (Merck, Darmstadt, Germany) and 0.01 M NaH_2PO_4 (Sigma Aldrich, St Louis, MO, USA) was used to prepare trypsin, thermolysin and papain solutions. Pepsin A solution was prepared in citrate buffer (pH 3.0) consisting of 0.1 M citric acid (Sigma Aldrich) and 0.1 M sodium citrate (Sigma Aldrich). Protease from pancreas bovine was dissolved in PBS (pH 7.4).

The wash buffer was PBS (pH7.4) with 0.05 % Tween 20 (v/v). The streptavidin-HRP solution was prepared using PBS (pH 7.4). The substrate buffer (pH 5.0) was composed of 0.1 M sodium acetate (Sigma Aldrich) and 0.2 % (v/v)

citric acid (Sigma Aldrich). To prepare the substrate solution, 6 mg of TMB was dissolved in 1 mL of DMSO and 332 µL of this solution was added to 10 mL of substrate buffer plus 3 µL hydrogen peroxide. The stop solution consisted of 1.0 or 2.0 M H_2SO_4 (Lamers & Pleuger B.V., Hertogenbosch, Netherlands).

3.2.2. Assay in microplates

The developed assay on solid phase for detecting proteases was based on four steps: coating, blocking, proteolysis and detection. First, microplate was coated with 100 µL of biotinylated casein (BC) solution added in each well. Three wells were filled with PBS (i.e. not coated) and considered to calculate background. Plate was sealed and incubated at 37 °C for 2 h. After three washing steps, unoccupied sites were blocked by adding 200 µL of blocking buffer following by incubation at 37 °C for 1 h. Next, 100 µL of solution containing protease was added to each well. When proteases diluted in buffer were used, plates were incubated at 37 °C for 2 h. However, for detecting proteases in milk samples, incubation step was performed overnight. Subsequently, microplate was washed three times and 100 µL streptavidin-HRP was added to wells corresponding the detection step. Streptavidin-HRP was bound to remained biotin molecules during an incubation step at 37 °C for 2 h. Plate was washed five times and wells were filled with 100 µL TMB substrate. Subsequently, the microplate was incubated at 37 °C for 30 min. Finally, 100 µL of stop solution was added and absorbance was measured at 450 nm in Multiskan Ex (Thermo Fisher Scientific, Vantaa, Finland). These steps were used throughout this study.

3.2.3. Parameters and settings

Several parameters were analyzed in order to develop a sensitive method: blocking buffer (Protein-free T20 blocking buffer or SmartBlock^{TM}), BC concentration (from 10.0 to 0.007 ng µL-1), streptavidin-HRP dilution (from 1:10,000 to 1:50,000) and stop solution concentration (1.0 M or 2.0 M H_2SO_4). All these parameters were evaluated using assay described in Section 2.2. Absorbance values and background were considered for parameters comparison. Background was calculated dividing absorbance of non-coated well by maximum absorbance.

3.2.4. Protease detection in buffer

Different concentrations of trypsin, papain, pepsin, thermolysin and protease from bovine pancreas were prepared using the appropriate buffer as described in Section 2.1. Stock solution of these five enzymes was made dissolving 1.0 mg of each protease in 1.0 mL buffer based in the solubility of each enzyme. Every stock solution was diluted in appropriate buffer serially until dilution 10^{-7}. Assay was performed using eight different concentrations of each protease in a dilution range from 1:1 to 1:100000000.

3.2.5. Protease detection in milk samples

3.2.5.1. Removing of casein by precipitation

To prevent competition between BC coated to plate and casein present in milk samples, HCl (5.0 M) was added to 30 mL of full fat UHT milk until reaching pH 4.5. As pI of casein is 4.6, this protein precipitates at pH 4.5. Casein was separated from supernatant by centrifugation at 5000 *g* for 5 min. The pH of supernatant was raised up to 6.9 – 7.0 by adding NaOH (5.0 M).

3.2.5.2. Protease dilution before casein precipitation

Thermolysin was added in 40 mL of full fat UHT milk in different concentrations (10 – 1.0 x 10^{-3} mU μL-1) and solutions were placed on ice. Acidification and neutralization, as described in Section 2.5.1., were performed to precipitate casein. Thermolysin dilutions were also made in phosphate buffer and in milk supernatant (100 – 1.0 x 10^{-4} mU μL-1). Proteolytic activity of papain and thermolysin diluted in phosphate buffer (pH 6.5) and supernatant was measured by microplate assay. Proteolytic activity was measured in duplicate by microplate assay.

3.2.5.3. Protease from psychrotrophic bacteria

An aliquot of 100 μL of stock solution of *S. liquefaciens* strains L53, L79, L104 and *P. fluorescens* L227 isolated from Brazilian refrigerated raw milk (Machado et al., 2015), which was stored at – 80 °C, was added to 5 mL Brain Heart Infusion (BHI) (Oxoid, Basingstoke, England). After overnight incubation at 22 °C, 500 μL of grown culture was added to 5 mL full fat UHT milk. Milk was incubated

at 22 °C for 24 h and 10 μL of inoculated milk was added to 50 mL of fresh full fat UHT milk. Milk samples were stored at 7 °C for 4 days, containing approximately 10^8 CFU mL^{-1} of *S. liquefaciens* or *P. fluorescens*, and heated at 95 °C for 8.45 min (Marchand et al., 2008). As a positive control, thermolysin was added to a fresh UHT milk sample. From all milk samples, casein was precipitated by acidification. Supernatant was serially diluted up to 10^{-5} and proteolytic activity was measured by microplate assay.

3.3. Results

3.3.1. Parameters settings

Several parameters were evaluated to optimize the assay on solid phase for detecting proteases. Selected blocking buffer, BC concentration, streptavidin-HRP dilution and stop solution concentration resulted in a higher signal and a lower background. In all assays that followed this evaluation, plates were coated using 0.07 ng μL^{-1} of BC solution because this concentration was small enough to result in a detectable difference in absorbance when proteolytic activity of papain in small concentrations (from 3.1 x 10^{-4} to 3.1 mU μL^{-1}) was measured. When BC concentrations lower than 0.07 ng μL^{-1} were used for coating plates, the signal captured by the absorbance reader was so low that proteolytic activity could not be measured.

Remained biotin was detected by adding a streptavidin-HRP solution in a dilution of 1:25,000 because this dilution produced the highest signal, but when wells were not coated with BC, the signal was low enough to obtain a low background.

There was no difference in absorbance values or background when different blocking buffers or stop solutions were used. Thus, to reduce costs, Protein-free T20 blocking buffer was used in blocking step and H_2SO_4 1 M was selected for stopping reaction.

3.3.2. Protease in buffer

Although BC coated onto the surface of the wells was used as a substrate for all tested enzymes (Table 1), sensitivity and background depend on the enzyme used for hydrolysis of BC. Trypsin presented the lowest proteolytic activity

measured by microplate assay, if sensitivity and background are considered and compared to pepsin, papain, thermolysin and protease from bovine pancreas values, while papain was the most effective protease in BC hydrolysis.

Table 1 - Hydrolysis of biotinylated casein by different proteases.

Enzyme	Activity[a]	Sensitivity[b] (mU μL^{-1})	Background[c]	Range for assay (mU μL^{-1})
Trypsin	10,000 BAEE U/mg protein	90.0	65.6 %	0.001 – 10000
Pepsin A	355 U/mg solid	0.213	31.6 %	0.000035 - 350
Papain	3.10 BAEE U/ mg solid	0.00031	14.7 %	0.00000031– 3.1
Thermolysin	100 U/mg protein	0.04	28.2 %	0,00001 - 100
Protease from bovine pancreas	5 U/mg solid	0.8	42.8 %	0.0000005 – 5.0

[a] Units of activity was defined by supplier.
[b] Sensitivity was determined as the activity required to decrease from 100 % to 90 % the percentage of maximum absorbance.
[c] The absorbance measured after hydrolysis by the most concentrated enzymatic solution (1.0 mg enzyme mL^{-1}) was divided by the maximum absorbance to calculate background.

3.3.3. Protease in milk samples

3.3.3.1. Protease dilution after casein precipitation

Casein, which could influence BC detection by microplate assay, was removed from milk samples after acidification. Milk samples after precipitation were used to dilute thermolysin and papain for evaluation of influence of casein from milk samples in microplate assay.

The slope of sigmoidal curve, which represents the thermolysin activity, in the supernatant was close to the curve of this enzyme diluted in buffer (Figure 1). Thus, the casein precipitation of milk samples improved thermolysin detection by microplate assay once the enzyme was added after acidification and neutralization. However, when papain activity curves are considered, the dilution in buffer and in supernatant resulted in two different slopes (Figure 1). Microplate method was more sensible when papain was diluted in buffer. Precipitation can be an efficient treatment for milk samples analyses, but it depends on the tested protease.

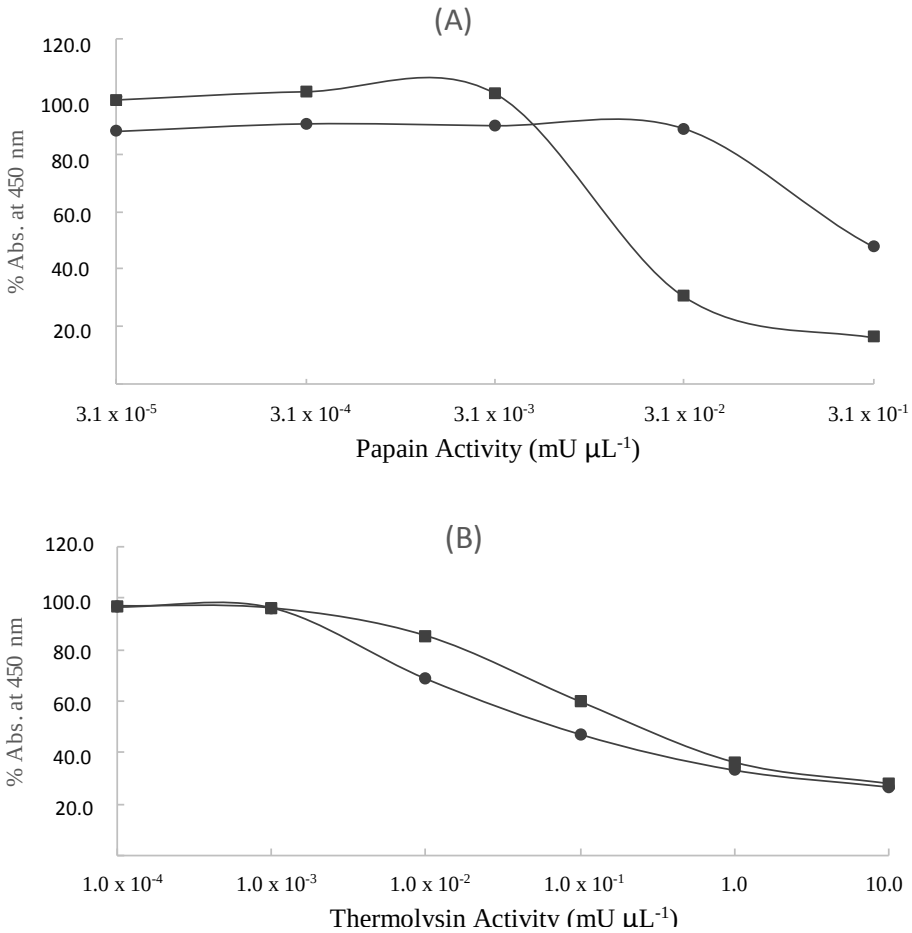

Figure 1 – Interference of casein in protease detection by microplate assay. Papain (A) and thermolysin (B) were serially diluted in buffer (squares) and supernatant (circles).

3.3.3.2. Protease dilution before casein precipitation

Casein precipitation was an efficient step in sample preparation before analysis by microplate assay. However, acidification followed by neutralization could affect enzymatic activity of proteases in milk samples. To assess the consequences of casein precipitation in proteolytic activity, thermolysin was added in milk samples after and before precipitation. Proteolytic activity of thermolysin diluted in milk samples were compared to thermolysin dissolved in buffer. When thermolysin was added to the supernatant after precipitation, the slope of curve was similar to the slope when enzyme was diluted in buffer (Figure 2). Nevertheless, when thermolysin (10 - 0.001 mU μL^{-1}) was added before casein precipitation,

proteolytic activity was about 65.5 - 45.6 % of absorbance at 450 nm and no sigmoidal curve was detected (Figure 2). Furthermore, the sensitivity was extremely reduced, showing that the thermolysin detection is unfeasible when the enzyme is added to milk sample before casein precipitation. Casein precipitation had a considerable effect in thermolysin activity detection by microplate assay.

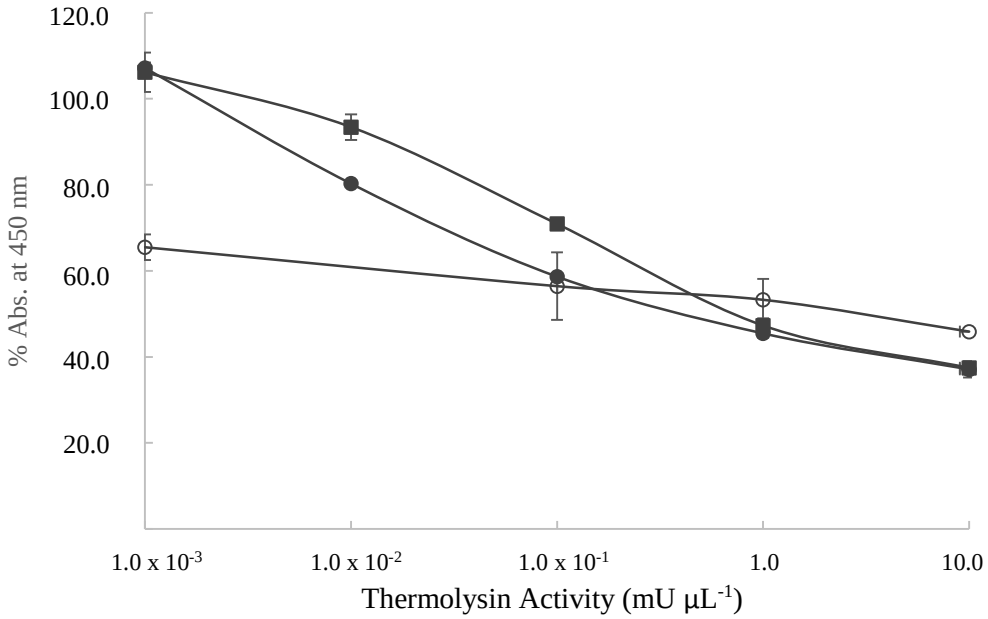

Figure 2 – Effect of casein precipitation on protease added in milk samples. Thermolysin was diluted in buffer (squares) and supernatant after casein precipitation (black circles) or in milk before casein precipitation (white circles). The standard deviation is represented by bars.

3.3.3.3. Protease from psychrotrophic bacteria

As thermolysin activity changed after casein precipitation, proteolytic activity from proteases produced by psychrotrophic microorganism was evaluated by adding them after acidification followed by neutralization. The sigmoidal activity curve presented in Figure 3 corresponds to thermolysin activity when the protease was diluted in supernatant after casein precipitation. The curves that represents proteolytic activity of proteases from *S. liquefaciens* and *P. fluorescens* are not sigmoidal indicating there was no degradation of BC coated on plates. Thus, sample preparation by precipitation may not be used before protease detection by microplate assay.

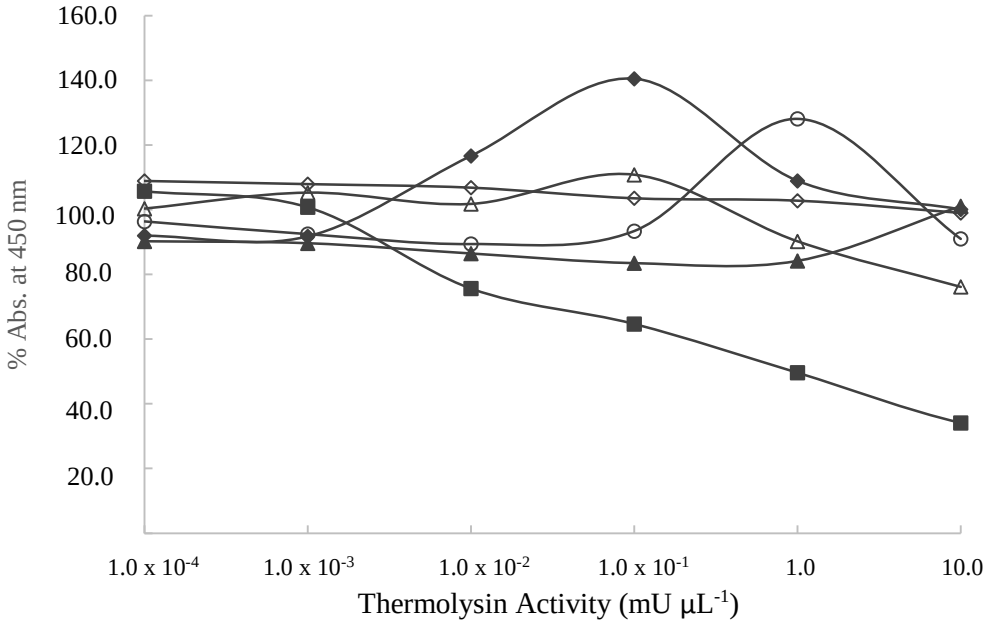

Figure 3 – Proteolytic activity of milk with thermolysin (white circles) or milk samples inoculated with *S. liquefaciens* L53 (black triangles), L79 (white triangles), L104 (white rhambus) and *P. fluorescens* L227 (black rhombus) comparing to thermolysin activity in buffer (squares).

3.4. Discussion

The microplate assay developed in this study reports a promising approach for detecting heat-resistant proteases in milk. The BC binding on a solid phase facilitates washing steps, while use of labeled substrate allows ready detection of remaining casein (Gan et al., 1999). Using this method, results may be achieved in 4 h 30 min, but it may be optimized for applying in dairy industry. Moreover, the microplate assay allows considerable flexibility in its design and therefore in its corresponding sensitivity by changing parameters settings.

Sensitivity of solid-phase assay differs according to the tested enzyme as evidenced in Table 1. Probably, this difference in sensitivity is related to target site of each enzyme. Trypsin, papain, pepsin and thermolysin belong to serine, cysteine, aspartic and metalloprotease classes, respectively. Protease from bovine pancreas is a mixture of different enzymes wherefore it is not classified in these four classes. These four mechanistic classes may be separated in two different groups: those that form covalent enzyme complexes as serine and cysteine proteases and those that do not form covalent complexes as aspartic and metalloproteases (Dunn, 2001). This

distinction based on strategy for hydrolysis catalyzing associated with the fact that target site for different enzymes are not the same can explain the difference on sensitivity when enzymes from distinct groups were tested. Furthermore, this assay on solid phase may represent a limitation for proteases that require significant folding in substrate (Sarath et al., 2001). As the main application of this analytical method is the detection of heat-resistant metalloproteases produced by psychrotrophic microorganisms, the next step for assay optimization would be the measurement of proteolytic activity of a purified metalloprotease from psychrotrophic microorganisms isolated from milk samples. Another factor that may be monitored is biotinylation since biotin molecules may represent a steric hindrance to protease attack depending on the site of attachment to casein. There are several strategies for protein biotinylation that may be used for casein labeling (Elia, 2010; Lue et al., 2004).

As milk is a complex food matrix, which contains components that can compromise protease detection (Figure 3), microplate assay has to be improved for milk analysis although it was an efficient method to measured proteolytic activity in buffer. In this study, since casein may interfere on protease measurement, this protein was removed from milk samples by precipitation, but, as highlighted in Figure 2, acidification followed by neutralization affected proteolytic activity when protease was added to milk samples before precipitation besides the inefficient detection of proteases from psychrotrophic microorganisms. Thus, approaches to remove milk components, which can interfere on protease analysis, may be evaluated and improved so that the microplate assay may be applied in dairy industry as a tool for quality control.

3.5. Conclusions

The microplate assay developed in this study would be useful for detecting proteases in low concentration sufficient to destabilize dairy products. However, steps as casein biotinylation and sample preparation should be improved before its application in dairy industry although it is a simple and amenable to automation method.

3.6. References

Baglinière, F., Tanguy, G., Jardin, J., Matéos, A., Briard, V., Rousseau, F., Robert, B., Beaucher, E., Humbert, G., Dary, A., Gaillard, J.L., Amiel, C., Gaucheron, F., 2012. Quantitative and qualitative variability of the caseinolytic potential of different strains of *Pseudomonas fluorescens*: Implications for the stability of casein micelles of UHT milks during their storage. Food Chemistry 135, 2593-2603.

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

Dunn, B.M., 2001. Determination of protease mechanism, In: Beynon, R., Bond, J.S. (Eds.), Proteolytic Enzymes, 2nd ed. Oxford University Press, Oxford, p. 340.

Elia, G., 2010. Protein biotinylation, In: Coligan, J.E., Dunn, B.M., Ploegh, H.L., Speicher, D.W., Wingfield, P.T. (Eds.), Current Protocols in Protein Science. John Wiley & Sons, Inc., pp. 3.6-3.6.21.

Gan, Z., Marquardt, R.R., Xiao, H., 1999. Protease and protease inhibitor assays using biotinylated casein coated on a solid phase. Analytical Biochemistry 268, 151-156.

Ledenbach, L.H., Marshall, R.T., 2010. Microbiological spoilage of dairy products, Compendium of the Microbiological Spoilage of Foods and Beverages. Springer, pp. 41-67.

Lue, R.Y.P., Chen, G.Y.J., Hu, Y., Zhu, Q., Yao, S.Q., 2004. Versatile protein biotinylation strategies for potential high-throughput proteomics. Journal of the American Chemical Society 126, 1055-1062.

Machado, S.G., Bazzolli, D.M.S., Vanetti, M.C.D., 2013. Development of a PCR method for detecting proteolytic psychrotrophic bacteria in raw milk. International Dairy Journal 29, 8-14.

Machado, S.G., Silva, F.L., Bazzolli, D.M.S., Heyndrickx, M., Costa, P.M.A., Vanetti, M.C.D., 2015. *Pseudomonas* spp. and *Serratia liquefaciens* as predominant spoilers in Brazilian cold raw milk. Journal of Food Science, *In press*.

Marchand, S., Coudijzer, K., Heyndrickx, M., Dewettinck, K., De Block, J., 2008. Selective determination of the heat-resistant proteolytic activity of bacterial origin in raw milk. International Dairy Journal 18, 514-519.

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

Martin, N.H., Ranieri, M.L., Murphy, S.C., Ralyea, R.D., Wiedmann, M., Boor, K.J., 2011. Results from raw milk microbiological tests do not predict the shelf-life performance of commercially pasteurized fluid milk. Journal of Dairy Science 94, 1211-1222.

Martins, M.L., de Araújo, E.F., Mantovani, H.C., Moraes, C.A., Vanetti, M.C.D., 2005. Detection of the *apr* gene in proteolytic psychrotrophic bacteria isolated from refrigerated raw milk. International Journal of Food Microbiology 102, 203-211.

Sarath, G., Zeece, M.G., Penheiter, A.R., 2001. Protease assay methods, In: Beynon, R., Bond, J.S. (Eds.), Proteolytic enzymes, 2nd ed. Oxford University Press, Oxford, p. 340.

Sørhaug, T., Stepaniak, L., 1997. Psychrotrophs and their enzymes in milk and dairy products: Quality aspects. Trends in Food Science & Technology 8, 35-41.

Vanetti, M.C.D., 2009. Spoilage Detection, In: Nollet, L.M.L., Toldrá, F. (Eds.), Handbook of Dairy Foods Analysis. CRC Press, Boca Raton, pp. 665-676.

GENERAL CONCLUSIONS

The quality of milk samples did not reach the requirements of Brazilian and European legislation. Psychrotrophic and *Pseudomonas* counts in raw milk samples reached high levels after cold storage simulation demonstrating the potential risk of milk spoilage. Regarding the psychrotrophic proteolytic microbiota, *S. liquefaciens* and *Pseudomonas* were identified as the dominant psychotrophs, which may produce heat-resistant proteases.

S. liquefaciens secretes only one protease encoded by *ser2* gene with, approximately, 52 kDa and heat resistance similar to AprX from *Pseudomonas* spp. considering the incubation conditions and the heat-treatment evaluated in this study. However, spoilage potential of *S. liquefaciens* is heterogeneous because Ser2 production is strain-dependent.

As several heat-resistant proteases could be present in milk samples, the developed assay on microtiter plates for detecting proteolytic activity would be useful in the dairy sector if the sensibility and accuracy were sufficient to detect heat-resistant proteases in low concentrations.