PROTEOLYTIC POTENTIAL OF PSEUDOMONAS FLUORESCENS ISOLATED FROM REFRIGERATED RAW MILK

Cláudia Lúcia Oliveira Pinto[1*], Solimar Gonçalves Machado[2], Rodrigo Rezende Cardoso[3], Rita Maria Alves[4], Maria Cristina Dantas Vanetti[2]

ABSTRACT – The growth rate and the proteolytic activity of *Pseudomonas fluorescens* strains 07A and 041, isolated from cow's milk, were evaluated at 2, 4, 7 and 10ºC. *P. fluorescens* promoted protein degradation during storage of milk samples as observed by Proteolytic activity measurement, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and heat stability of milk. Casein hydrolysis resulted in loss of thermal stability of milk and in formation of fragments of low and medium molecular mass. Temperatures up to 10°C did not guarantee raw milk quality when contamination by *P. fluorescens* was equal or higher than 10^6 cfu/mL.

Keywords: casein hydrolysis, milk spoilage, protease, psychrotrophic.

POTENCIAL PROTEOLÍTICO DE PSEUDOMONAS FLUORESCENS ISOLADA DE LEITE CRU REFRIGERADO

RESUMO – A taxa de crescimento e a atividade proteolítica das estirpes 07A e 041 de Pseudomonas fluorescens, *isoladas a partir de leite cru de vacas, foram avaliadas após incubação a 2, 4, 7 e 10°C. P. fluorescens promoveu a degradação de proteínas durante armazenamento de amostras de leite, como observado pela determinação da atividade proteolítica, por eletroforese em gel de poliacrilamida desnaturante (SDS-PAGE) e pela estabilidade térmica do leite. A hidrólise da caseína resultou na perda da estabilidade térmica do leite e na formação de fragmentos de baixa e média massa molecular. Temperaturas de até 10°C não garantiram a qualidade do leite cru quando a contaminação por* P. fluorescens *foi igual ou superior a 10^6 UFC/mL.*

Palavras chave: deterioração do leite, hidrólise da caseína, psicrotróficos, protease.

1. INTRODUCTION

The degradation of the casein fraction in milk may be caused by native or microorganism-produced protease (Nielsen, 2002; Hachana et al., 2010) and the proteolysis in milk has gained much interest due to its complexity and variable effects on quality of dairy products (Adams et al., 1976; Larson et al., 2006; Teh et al., 2012). These enzymes are associated with technological problems, including coagulation, gelation, formation of off flavors, loss quality and reduced shelf life of UHT milk and other dairy products (Celestino et al., 1997; Sorhaug & Stepaniak, 1997) besides economic problems in the dairy industry.

The practice of extended refrigerated storage of raw milk on the farm, during transportation and at the dairy plant have led to a selection of psychrotrophic bacteria (Eneroth et al., 2000; Munsch-Alatossava & Alatossava, 2006; Ercolini et al., 2009; Rasolofo et al., 2010; De Jonghe et al., 2011; Raats et al., 2011). *Pseudomonas* is the predominant psychrotrophic genus isolated from refrigerated raw milk and a major spoilage agent in the dairy industry (Sorhaug & Stepaniak, 1997;

[1] Agriculture and Livestock Research Corporation's of Minas Gerais, EPAMIG, Viçosa, MG, Brasil. 2Department of Microbiology, Federal University of Viçosa, Viçosa, 36570-000, MG, Brazil.
[3] Department of Food Science and Technology, Federal University of Viçosa, Viçosa, 36570-000, MG, Brazil.
[4] Brazilian Agricultural Research Corporation's, Embrapa Wheat, Passo Fundo, RS, Brazil.
[*] Corresponding author: C.L.O. Pinto, claudia.epamig@gmail.com.

Marchand et al., 2009a, Decimo et al., 2014). The predominance of this genus is justified by the fact that they present shorter generating time between 0ºC and 7ºC and a minimum growth temperature, up to -10ºC (Sorhaug & Stepaniak, 1997).

Although most psychrotrophics are inactivated by the conventional thermal treatments employed in the dairy industries, such treatments have minor effects on their enzymes. The extracellular proteases produced by these microorganisms, which degrade milk protein during storage under refrigeration are usually thermostable, keeping their activity even after pasteurization and ultra-high temperature (UHT) treatments (Sorhaug & Stepaniak, 1997; Marchand et al., 2009b). According to Dogan and Boor (2003), 51% of *Pseudomonas* strains from dairy industries are producers of proteases. The heat-stable alkaline metalloproteases in *P. fluorescens* strains isolated from refrigerated raw milk are encoded by *aprX* gene (Marchand et al., 2009b; Martins et al., 2005).

This study investigated *P. fluorescens* growth in milk stored at 2, 4, 7 and 10°C up to 10 days and its relationship to the proteolytic activities using a colorimetric method and polyacrylamide gel electrophoresis.

2. MATERIAL AND METHODS

2.1. Bacterial strains used and culture conditions

P. fluorescens strains 07A and 041 used in this study were isolated from cold raw milk samples collected from refrigeration tanks at the production site and identified by Pinto et al. (2006). Culture stocks were prepared in Luria-Bertani Broth (LB) (Oxoid, Hampshire, England) containing 20% (v/v) sterile glycerol (VETEC Química Fina Ltda., Xerém, Brazil) and were frozen at -80°C. Before each experiment, the cells were cultured by two consecutive times in skim milk powder 12% (w/v) for 24 h at 25°C.

2.2. *P. fluorescens* growth at low temperatures

Samples of 6 mL of skim milk powder 12% (w/v) were sterilized at 121°C for 15 min and inoculated in duplicate with, approximately, 10^4 cfu/mL of each *P. fluorescens* strain (07A and 041). Samples incubated at 4, 7 and 10°C were analyzed every 24 h. When samples were incubated at 2°C, the analysis was performed every 48 h, because growth rate is lower for smaller temperature. The number of viable *P. fluorescens* cells in milk samples was determined with the microdroplet method (Morton, 2001) in nutrient agar. This experiment was repeated three times. The specific growth rate (μ), the generation time (g) and the maximum population density (MPD) were determined for each strain and temperature (Brock et al., 1994).

2.3. Proteolytic activity and thermal stability of milk

Samples prepared as described in item 2.2 were subjected to proteolytic activity analysis determined in terms of mg tyrosine released/5 mL milk according to the method of Hull (1947) and thermal stability test in water bath at 72°C for 15 s when clot formation was observed.

2.4. Electrophoretic profile of casein degradation by *P. fluorescens*

Raw milk samples were obtained from healthy cow belonging to a barn of the Federal University of Viçosa, MG, Brazil in accordance to hygienic production practice, collected in sterilized recipients, transported under refrigeration to the laboratory for analysis and use in the experiments.

Raw milk samples were plated in Standard Methods Caseinate Agar SMCA for viable psychrotrophic and psychrotrophic proteolytic bacteria counts, performed after incubation at 6.5 ± 0.5°C for 10 days as recommended by Frank et al. (1992). *Pseudomonas* spp. count was performed 72 h after incubation, at 22°C, in agar base for *Pseudomonas* CM559 (Oxoid, Hampshire, England), with addition of selective supplement (Oxoid, SR 103E, Hampshire, England) contained cetrimide (10.0 mg/L), fuccidine (10.0 mg/L) and cephalosporin (50.0 mg/L).

Raw milk samples of 100 mL were inoculated with, approximately, 10^6 cfu/mL of *P. fluorescens* 07A or 041 and incubated at 2, 4, 7, and 10°C. Immediately after inoculation and following 1, 2, 4 and 6 days of storage, samples were collected for determination of the growth of *P. fluorescens* strains and the effect of its protease on the casein fractions. The viable number of *P. fluorescens* 07A and 041 was determinate on SMCA at 22°C incubation.

The effects of bacterial protease on the milk proteins were evaluated by sodium dodecyl sulphate polyacrylamide gel electrophoresis – SDS-PAGE with

modifications of the Laemmli method, in a Bio-Rad system (Bio-Rad, Mini-Protean® II, Richmond, USA) with a Bio-Rad source (Bio-Rad, Power Pac 300, Richmond, USA). The samples for milk protein analysis were prepared as described by Adams *et al*. (1976). Briefly, 10 mL aliquots of the refrigerated milk were acidified to pH 4.0 under constant mixing using 10 mol/L hydrochloric acid and later centrifuged for 10 min at 12,100 g in a Sorvall centrifuge (Du Pont Company, model RC 5C, Delaware, USA), at 4ºC. Serum was eliminated and following casein precipitation, the original volume was reconstituted with 100 mM Tris-HCl at a final pH of 6.9.

Separation gels of 15% (w/v) acrylamide capped by stacking gels of 4.5% (w/v) acrylamide were used. The final concentrations of Tris-HCl buffer and SDS in the separation and stacking gels were those recommended by Laemmli (1970). The gels were chemically polymerized by the addition of 0.025% (v/v) tetramethyl ethylene diamine (TEMED) and 0.05% (w/v) of ammonium persulphate. The electrode buffer (pH 8.6) contained Tris-base, glycine and SDS in the concentration used by Laemmli (1970). Samples were adjusted for concentration of approximately 10 mg/mL of protein (Laemmli, 1970). Prior to analysis, sample aliquots of 2 mL, corresponding to approximately 20 mg protein, were added 25 mL of the sample buffer solution and submitted to heat by immersion in boiling water during 3 min. Electrophoresis was conducted at 60 V for 20 min for staking and 80 V for 2 h, for separation of the protein fractions. The gels were stained for 2 h at room temperature in solution of 0.2% (w/v) Coomassie Brillant blue R in methanol: acetic acid: water (1:2:17 v/v) and distained by repeated washing in methanol: acetic acid: water (1:2:17 v/v).

Caseinic fractions were identified by analysis of individual samples of purified bovine milk a, b and k-casein standards (Sigma, Chemical Co, St Louis, USA) and low and medium range molecular mass standards (Promega Corporation, Madison, USA). The casein standards were prepared by dissolution of 1.0 mg in 1 mL of sample buffer and heated in boiling water for 3 min.

2.5. Statistical analyses

Bacterial counts were transformed to log counts before statistical analysis. Several parameters, such as maximum population density, specific growth rate, generation time, population and proteolysis values corresponding to loss of thermal stability of milk samples inoculated with *P. fluorescens* 07A and 041, were compared by using the Tukey-test at the 5% probability level. Statistical analysis was performed using the R software (R Core Team, 2013).

3. RESULTS

3.1. *P. fluorescens* growth at low temperatures

Representative data on the growth of *P. fluorescens* 07A and 041 in raw milk at different temperatures are shown in Table 1. As the temperature rose, the specific growth rate of both strains gradually increased. Both strains reach population above 10^9 cfu/mL at 10ºC after 4 days incubation. The temperature rise resulted in an increase of maximum population density achieved by *P. fluorescens* 07A and 041 evidenced by statistical analyses showed in Table 1.

An increase in growth temperature increased specific growth rate of *P. fluorescens* 07A and 041 (Table 1). Consequently, the duration of both lag phase (Figure 1) and generation time (Table 1) decreased. Both strains grew up to a 4 log increase at 2°C after 10 days. At 4°C, the strains 07A and 041 populations reached 10^8 cfu/mL after eight and six days, respectively (Figure 1), while at 7°C, this population was achieved after four and five days, respectively. A population of 10^9 cfu/mL was observed for 07A and 041 after seven days of storage at 10°C, corresponding to an increase of five logarithmic cycles.

3.2. Proteolytic activity and thermal stability of milk

The amount of tyrosine released, which is result of proteolysis, varied from strain 07A to strain 041, being the last one more proteolytic (Figure 1). As well as growth rate, proteolytic activity is also influenced by temperature (Figure 1). During storage at 2°C, proteolysis was slow and increased after six and eight days at 4°C, when milk was inoculated with *P. fluorescens* 41 and 07A, respectively.

The relationship among the population of the two strains of *P. fluorescens* and the degree of proteolysis during storage of milk samples was monitored by plating and proteolytic activity measurement. It was found that the amount of tyrosine remained constant until the bacterial population reached 10^8 cfu/mL. The increase in the amount of tyrosine coincided with the start of

Table 1 - Growth kinetic parameters of *P. fluorescens* 07A and 041 in skim milk incubated at cold temperatures

Temperature (°C)	Generation time(*g*, h)		Specific growth rate ($\mu_{maximum}$, h-1)		Maximum populationdensity (log_{10} cfu/mL)	
	07A	041	07A	041	07A	041
2	12.95 aA	10.97 bA	0.02 aA	0.03 aA	7.64 aA	8.69 bA
4	9.15 aB	8.05 bB	0.03 aA	0.04 aA	8.26 aB	8.92 bB
7	6.25 aC	6.19 bC	0.05 aB	0.05 aB	8.64 aC	8.85 aB
10	4.67 aD	4.59 bD	0.07 aC	0.07 aC	9.10 aD	9.28 aB

The same lowercase letters in lines and the same capital letters in columns indicate no significant difference between strains and among temperatures, respectively (PÃ0.05).

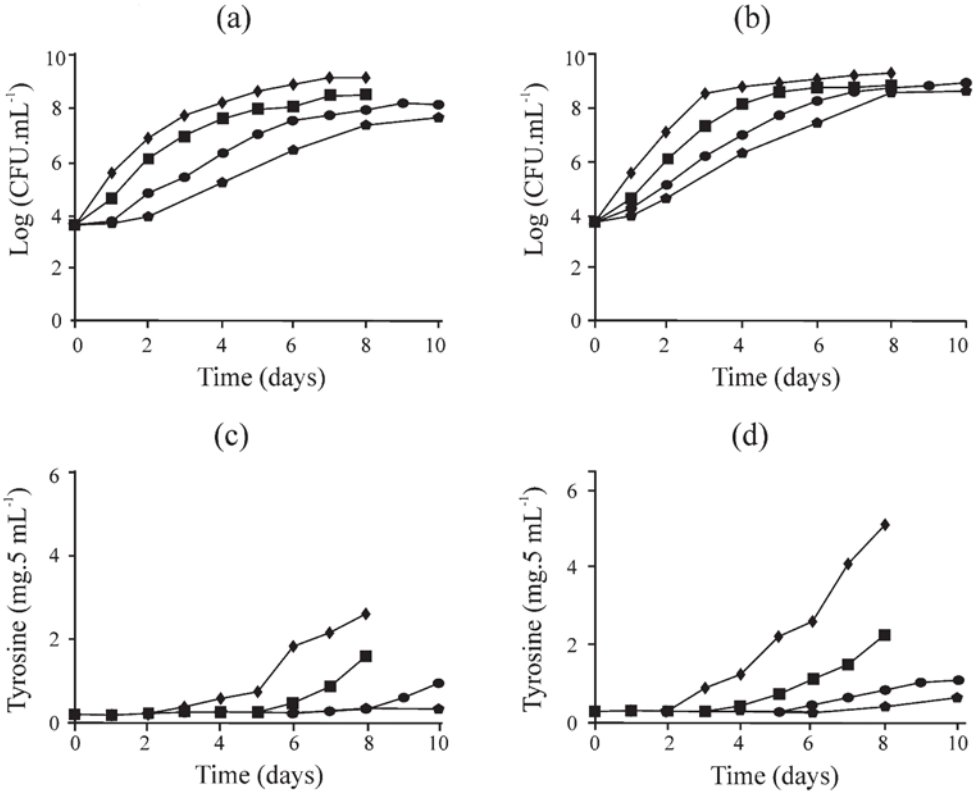

Figure 1 - *P. fluorescens* growth (a, b) and tyrosine concentration (c, d) during storage of skim milk inoculated with *P. fluorescens* 07A (a, c) and 041 (b, d) at different cold temperatures: 2°C (⬟), 4°C (●), 7°C (■) and 10°C (◆).

the stationary growth phase at all temperatures evaluated (Figure 1).

The stability to heat treatments at 72°C for 15 s was higher in samples stored at 2 and 4°C than for samples stored at 7 and 10°C (Table 2). Milk samples with a population exceeding 10^7 cfu/mL were showed more instability during heating at 72°C for 15 s (Table 2). However, population of *P. fluorescens* needed to destabilize milk samples was significantly different (P<0.05) when both strains were compared. *P. fluorescens* 041 was able to destabilize milk samples when reached, approximately, 10^8 cfu/mL while milk samples inoculated with *P. fluorescens* 07A were considered instable when this strain reached 10^7 cfu/mL (Table 2). Once reached temperatures higher than 10^7 - 10^8 cfu/mL, the amount of tyrosine released by both strains of *P. fluorescens* was statistical equal (P>0.05) regardless temperature of growth except for *P. fluorescens* 041 growing at 10°C, which presented a level of proteolysis much higher (Table 2).

3.3. Proteolysis of casein fractions

The initial psychrotrophic and *Pseudomonas* number in samples of raw milk aseptically collect was lower than 1 cfu/mL and no increase of this population over the 6 days incubation at 2 and 4ºC was verified. However, in samples maintained at 7ºC, the psychrotrophic number was 2.2 x 10^2 cfu/mL after 6 d. When maintained at 10ºC, the psychrotrophic population reached 6.6 x 10^2 cfu/mL after 4 days and 6.3 x 10^4 cfu/mL after 6 days. No alterations were confirmed on the electrophoresis profile of the proteins in the raw milk samples aseptically collected, non-inoculated and incubated at 2, 4, 7, and 10ºC, over a period of up to 6 days (Figure 2).

In inoculated milk, casein hydrolysis was verified through formation of fragments of low and medium molecular mass and, was accelerated as temperature increase (Figures 3 and 4). The growth of *P. fluorescens* 07A and 041 isolates in milk affected casein fractions, in different way and initially, κ-casein was hydrolyzed, with release of the band correspondent to *para*-κ-casein.

Table 2 - Relationship among *P. fluorescens* population, proteolysis and storage time for loss of thermal stability

Temperature (°C)	Population (log_{10} cfu/mL)		Proteolysis (mg tyrosine/5 mL)		Storage time for loss of thermal stability (72°C/15 s)	
	07A	041	07A	041	07A	041
2	7.33 aA	8.53 bAB	0.3474 aA	0.3530 aA	8	8
4	7.73 aA	8.33 bAB	0.3017 aA	0.3499 aA	7	6
7	7.66 aA	8.08 bB	0.2559 aA	0.2767 aA	3	3
10	7.67 aA	8.63 bA	0.3503 bA	0.7907 aB	3	3

The same lowercase letters in lines and the same capital letters in columns indicate no significant difference between strains and among temperatures, respectively (P>0.05).

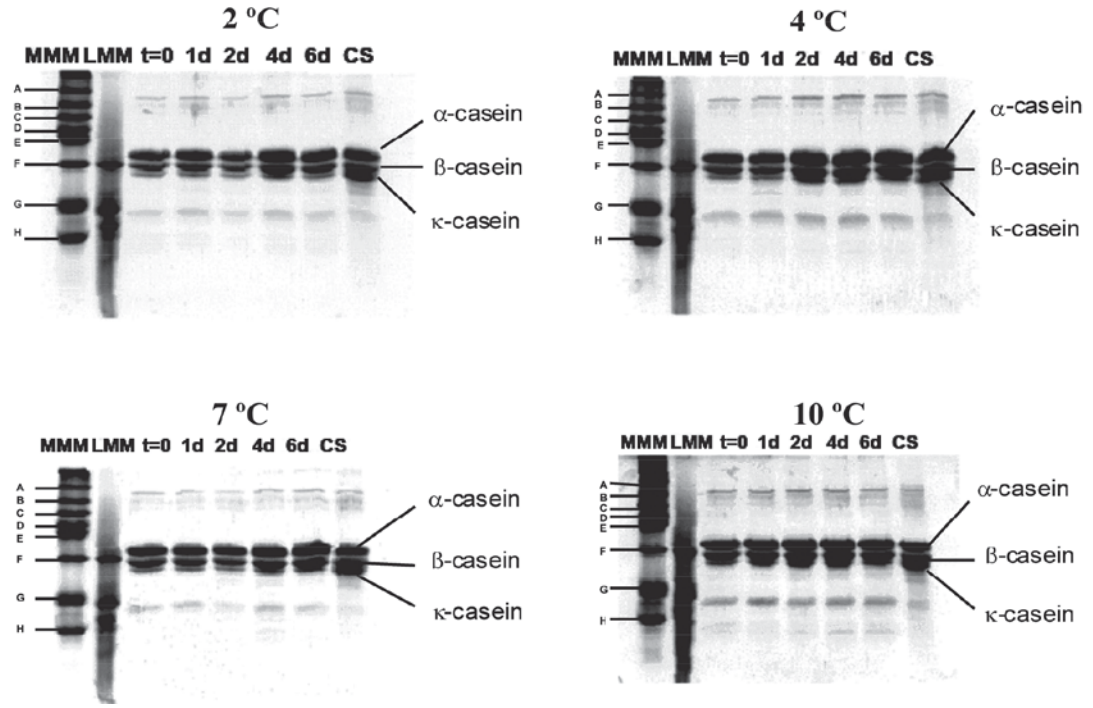

Figure 2 – SDS-PAGE (15%) of the proteins of the raw milk, incubated at 2, 4, 7 and 10ºC, and sprayed with Coomassie-Blue. Channels: 1) MMM – medium molecular weight standard; 2) LMM – low molecular weight standard; 3t = 0 – Milk at zero time; 4) 1d – Milk after 1 day incubation; 5) 2 d – Milk after 2 days incubation; 6) 4 d – Milk after 4 days incubation; 7) 6 d – Milk after 6 days incubation; 8) PC – Molecular weight standard of the casein fractions.

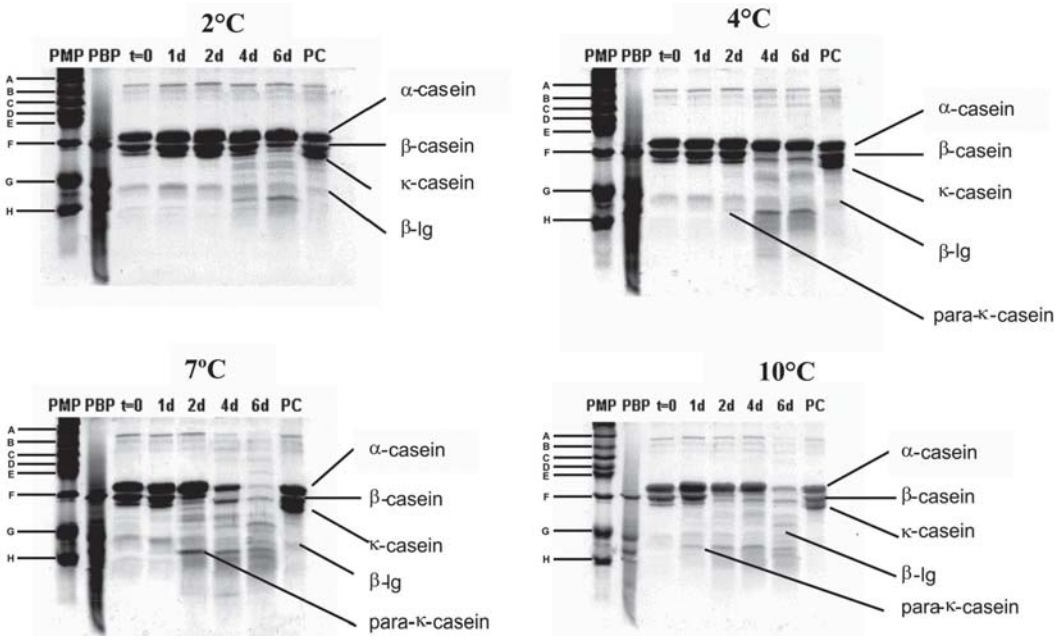

Figure 3 - SDS-PAGE (15%) of the proteins of raw milk inoculated with *P. fluorescens* 07A, incubated at 2, 4, 7 and 10ºC, and sprayed with Coomassie-Blue. Channels: 1) MMM – medium molecular weight standard; 2) LMM – low molecular weight standard; 3) t = 0 – Milk inoculated at zero time; 4) 1d – Milk inoculated after 1 day incubation; 5) 2 d – Milk inoculated after 2 days incubation; 6) 4 d – Milk inoculated after 4 days incubation; 7) 6 d – Milk inoculated after 6 days incubation; 8) PC – Molecular weight standard of the casein fractions.

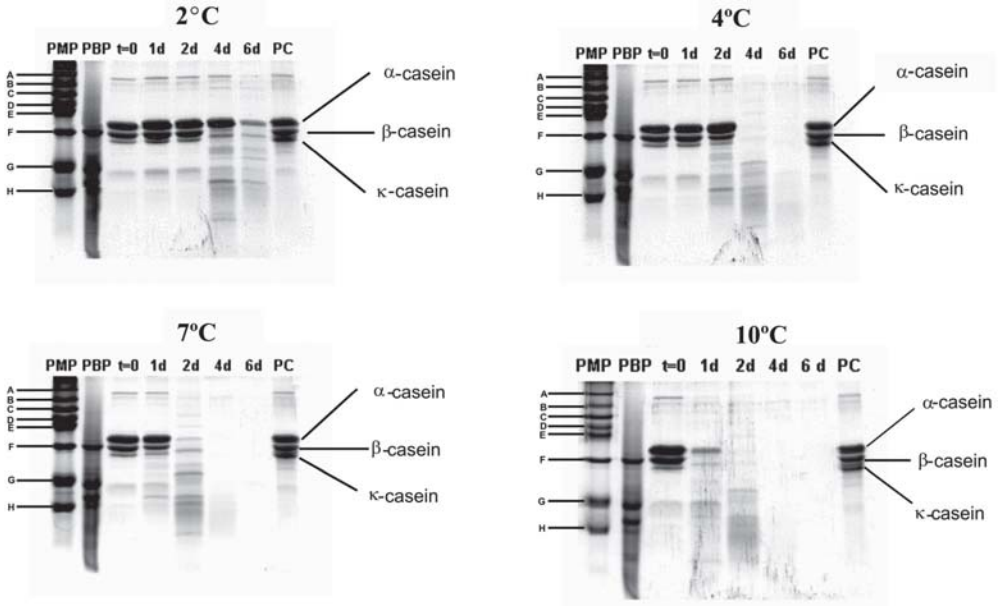

Figure 4 – SDS-PAGE (15%) of the proteins of raw milk inoculated with *P. fluorescens* 041, incubated at 2, 4, 7 and 10ºC, and sprayed with Coomassie-Blue. Channels: 1) MMM - Medium molecular weight standard; 2) LMM – Low molecular weight standard; 3) t = 0 – Milk inoculated at zero time; 4) 1 d – Milk inoculated after 1 day incubation; 5) 2 d – Milk inoculated after 2 days incubation; 6) 4 d – Milk inoculated after 4 days incubation; 7) 6 d – Milk inoculated after 6 days incubation; 8) PC – Molecular weight standard of the casein fractions.

Besides κ-casein, β-casein fraction was rapidly degraded, as observed with the reduced band intensity in SDS-PAGE (Figures 3 and 4).

At 2°C, alterations in k-casein fraction were confirmed in the inoculated samples (Figures 3 and 4), indicating that, even at temperatures as low as 2°C, hydrolysis of the casein fractions may occur along the storage period. Temperature increase to 4°C accelerated b and κ-casein degradation, with complete hydrolysis of all the casein fractions being observed on the fourth day of storage in milk inoculated with *P. fluorescens* 041, and on the sixth day, in milk inoculated with *P. fluorescens* 07A (Figures 3 and 4). Storage of raw milk samples individually inoculated with the *P. fluorescens* 07A and 041 at 7ºC and 10°C accelerated the degradation of the casein fractions. After 48 h, at 7°C, degradation of κ and β-casein fractions was observed, in addition to the appearance of the *para*-κ-casein band.

4. DISCUSSION

The *P. fluorescens* strains inoculated in skim milk reached numbers higher than 10^6 cfu/mL after 48 h incubation at 7°C (Figure 1). Although Kumaresan et al. (2007) reported that degradation of raw milk due to proteolytic activity was observed when psychrotrophic concentrations were above 10^6 cfu/mL, this study demonstrated that the amount of tyrosine remained constant until the bacterial population reached 10^8 cfu/mL. This difference can be explained by genetic diversity of psychrotrophic bacteria isolated from refrigerated raw milk (Martins et al., 2006). Baglinière et al. (20012) classified *P. fluorescens* strains in two groups according to their ability to destabilize UHT milk. These authors conclude that milk proteolysis was highly variable and strain-dependent, which were also proven by this study that demonstrated that population of *P. fluorescens* 041 necessary to destabilize milk samples was 1 log higher than population of *P. fluorescens* 07A.

The *P. fluorescens* population in milk stored at 7°C can reached 10^8 cfu/mL after 4 days incubation (Figure 1). Nero et al. (2009) reported that 12.2% of raw milk samples collected after milking had initial psychrotrophic numbers among 10^6-10^7 cfu/mL. After some days of refrigerated storage, psychrotrophic population achieved the stationary phase. According to Chen et al. (2003), protease production by psychrotrophic microorganisms occurs near the end of the logarithmic phase and progresses during the stationary phase.

The present study demonstrated that prolonged storage of milk at low temperatures, which were allowed by Brazilian law (Brasil, 2011), may result in a reduction of quality due to psychrotrophic protease activity. Furthermore, a high initial contamination by psychrotrophic proteolytic can result in economic losses to the dairy industry. The use of milk containing bacterial proteases may compromise the quality of dairy products, especially those with long shelf life, such as UHT milk.

The maintained of proteins integrity observed on SDS-PAGE in samples of raw milk aseptically collected indicate that the activity of indigenous proteases was not detectable during storage upon refrigeration up to 6 days (Figure 2). During cold storage of milk, plasmin may lose activity due to autolysis, and plasminogen may be activated, albeit probably at a slower rate than autolysis (Crudden et al., 2005).

It is hypothesized that psychrotrophic bacteria such as *Pseudomonas* spp. synthesize increased quantities of enzymes at low temperatures to compensate for decreased enzyme activity at such temperatures, in order to maintain their growth (Fairbairn & Law, 1986). Therefore, the development of a predictive model that combined microbial growth at temperature range of 2-20ºC with extracellular proteolytic and lipolytic enzyme activity of psychrotrophic bacteria, included *Pseudomonas* spp. could not be achieved since most enzymatic production and activity occurred during transition from exponential to stationary phase (Braun & Sutherland, 2003).

Although Baglinière et al. (2012) have observed the preferential proteolysis of β-casein, this study demonstrated that *P. fluorescens* 07A and 041 hydrolyze first κ-casein under low temperatures. Others studies also reported that proteases of psychrotrophic bacteria preferentially hydrolyzed caseins in the following order κ- > β- > αs-casein (Adams et al., 1976; Costa et al., 2002; Nicodème et al., 2005). This difference can be attributed to the differences in the conditions used in each study and in the strains used. In addition, casein fractions show different degrees of susceptibility due to their helicoidal form and aggregation as particles (Horne, 2006).

The degradation of milk proteins promoted by proteolytic activity of *P. fluorescens* showed the influence

of these spoilage bacteria on milk quality storage at low temperatures. The time elapsed between milking and processing should be reduced in order to prevent microbial growth and proteolytic activity. Furthermore, microbial contamination must be keep in low level with adoptions of good manufacture practices in order to minimize the alterations on milk quality.

5. CONCLUSIONS

Extensive degradation of caseins as a result of *P. fluorescens* growth observed in milk samples indicates that, even under refrigeration temperatures and storage time proposed in Brazilian legislation for raw milk storage, quality of milk can be compromised. Thus, it is important that good production practices are followed by milk producers to obtain a product with higher microbiological quality that can be conserved in storage conditions.

6. ACKNOWLEDGMENT

The authors thank the "Fundação de Amparo à Pesquisa do Estado de Minas Gerais" (FAPEMIG) for financial support.

7. CITED LITERATURE

ADAMS, D.M.; BARACH, J.T.; SPECK, M.L. Effect of psychrotrophic bacteria from raw milk on milk proteins and stability of milk proteins to ultrahigh temperature treatment. **Journal of Dairy Science**, v.59, n.5, p.823-827, 1976.

BAGLINIÈRE, F.; TANGUY, G.; JARDIN, J. et al. 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**, v.135, n.4, p.2593-2603, 2012.

BRASIL. 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, 2011.

BRAUN, P.; SUTHERLAND, J.P. Predictive modelling of growth and enzyme production and activity by a cocktail of *Pseudomonas* spp., *Shewanella putrefaciens* and *Acinetobacter*. **International Journal of Food Microbiology**, v.86, n.3, p.271-282, 2003.

BROCK, T.D. **Biology of microorganisms**. 7th Ed. New Jersey: Prentice Hall, 1994, 909p.

CELESTINO, E.L.; IYER, M.; ROGINSKI, H. 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**, v.7, n.2-3, p.129-140, 1997.

CHEN, L.; DANIEL, R.M.; COOLBEAR, T. Detection and impact of protease and lipase activities in milk and milk powders. **International Dairy Journal**, v.13, n.4, p.255-275, 2003.

COSTA, L.M.; GÓMEZ, F.S.; MOLINA, L.H.C. et al. Purificación y caracterización de proteasas de *Pseudomonas fluorescens* y sus efectos sobre las proteínas de la leche. **Archivos Latinoamericanos de Nutrición**, v.52, n.2, p.1-13, 2002.

CRUDDEN, A.; FOX, P.F.; KELLY, A.L. Factors affecting the hydrolytic action of plasmin in milk. **International Dairy Journal**, v.15, n.4, p.305-313, 2005.

DECIMO, M.; MORANDI, S.; SALVETTI, T. et al. Characterization of gram-negative psychrotrophic bacteria isolated from Italian bulk tank milk. **Journal of Food Science**, v.79, n.10, p.M2081-M2090, 2014.

DE JONGHE, V.; COOREVITS, A.; VAN HOORDE, K. et al. Influence of storage conditions on the growth of *Pseudomonas* species in refrigerated raw milk. **Applied and Environmental Microbiology**, v.77, n.2, p.460-470, 2011.

DOGAN, B.; BOOR, K.J. Genetic diversity and spoilage potentials among *Pseudomonas* spp. isolated from fluid milk products and dairy processing plants. **Applied and Environmental Microbiology**, v.69, n.1, p.130-138, 2003.

ENEROTH, A.; AHRNÉ, S.; MOLIN, G. Contamination routes of Gram-negative spoilage bacteria in the production of pasteurized milk, evaluated by randomly amplified polymorphic DNA (RAPD). **International Dairy Journal**, v.10, n.5-6, p.325-331, 2000.

FAIRBAIRN, D.J.; LAW, B.A. Proteinase of psychrotrophic bacteria: their production, properties, effects and control. **Journal of Dairy Research**, v.53, n.1, p.139-77, 1986.

FRANK, J.F.; CHRISTEN, G.L.; BULLERMAN, L.B. Tests for groups of microorganisms. In: MARSHALL, R.T. (Ed.). **Standard methods for the examination of dairy products**. New York: APHA, p.271-286, 1992.

HACHANA, Y.; KRAIEM, K.; PAAPE, M.J. Effect of plasmin, milk somatic cells and psychrotrophic bacteria on casein fractions of ultra high temperature treated milk. **Food Science and Technology**, v.16, n.1, p.79-86, 2010.

HORNE, D.S. Casein micelle structure: Models and muddles. **Current Opinion in Colloid & amp**; Interface Science, v.11, n.2-3, p.148-153, 2006.

HULL, M.E. Studies on milk proteins. II Colorimetric determination of the partial hydrolysis of the proteins in milk. **Journal of Dairy Science**, v.30, p.881-894, 1947.

KUMARESAN, G.; ANNALVILLI, R.; SIVAKUMAR, K. Psychrotrophic spoilage of raw milk at different temperatures of storage. **Journal of Applied Sciences Research**, v.3, n.11, p.1383-1387, 2007.

LAEMMLI, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. **Nature**, v.227, n.5259, p.680-685, 1970.

LARSON, N.K.; ISMAIL, B., NIELSEN, S.S. et al. Activity of Bacillus polymyxa protease on components of the plasmin system in milk. **International Dairy Journal**, v.16, n.6, p.586-592, 2006.

MARCHAND, S.; HEYLEN, K.; MESSENS, W. et al. Seasonal influence on heat-resistant proteolytic capacity of *Pseudomonas lundensis* and *Pseudomonas fragi*, predominant milk spoilers isolated from Belgian raw milk samples. **Environmental Microbiology**, v.11, n.2, p.467-482, 2009a.

MARCHAND, S.; VANDRIESCHE, G.; COOREVITS, A. et al. Heterogeneity of heat-resistant proteases from milk *Pseudomonas* species. **International Journal of Food Microbiology**, v.133, n.1-2, p.68-77, 2009b.

MARTINS, M.L.; ARAÚJO, E.F.; MANTOVANI, H.C. et al. Detection of the *apr* gene in proteolytic psychrotrophic bacteria isolated from refrigerated raw milk. **International Journal of Food Microbiology**, v.102, n.2, p.203-211, 2005.

MARTINS, M.L.; PINTO, C.L.O.; ROCHA, R.B. et al. Genetic diversity of Gram-negative, proteolytic, psychrotrophic bacteria isolated from refrigerated raw milk. **International Journal of Food Microbiology**, v.111, n.2, p.144-148, 2006.

MORTON, R.D. Aerobic plate count. In: DOWNES, F.P. and ITO, K. (Ed.). **Compendium of Methods for the Microbiological Examination of Foods**. 4th Ed. Washington: American Public Health Association, p.63-67, 2001.

MUNSCH-ALATOSSAVA, P.; ALATOSSAVA, T. Phenotypic characterization of raw milk-associated psychrotrophic bacteria. **Microbiological Research**, v.161, n.4, p.334-346, 2006.

NERO, L.A.; VIÇOSA, G.N.; PEREIRA, F.E.V. Qualidade Microbiológica do leite determinada por características de produção. **Ciência e Tecnologia de Alimentos**, v.29, n.2, p.386-390, 2009.

NICODÈME, M.; GRILL, J.-P.; HUMBERT, G. et al. Extracellular protease activity of different *Pseudomonas* strains: dependence of proteolytic activity on culture conditions. **Journal of Applied Microbiology**, v.99, p.641-648, 2005.

NIELSEN, S.S. Plasmin system and microbial proteases in milk: characteristics, roles and relationship. **Journal of Agricultural and Food Chemistry**, v.50, n.22, p.6628-6624, 2002.

PINTO, C.L.O.; MARTINS, M.L.; VANETTI, M.C.D. Qualidade microbiológica de leite cru refrigerado e isolamento de bactérias psicrotróficas proteolíticas. **Ciência e Tecnologia de Alimentos**, v.26, n.3, p.645-651, 2006.

RAATS, D.; OFFEK, M.; MINZ, D. et al. Molecular analysis of bacterial communities in raw cow milk and the impact of refrigeration on its structure and dynamics. **Food Microbiology**, v.28, n.3, p.465-471, 2011.

R CORE TEAM. R: A language and environment for statistical computing. 2013. In: http://www.R-project.org/ (accessed on January 4th 2015).

SØRHAUG, T.; STEPANIAK, L. Psychrotrophs and their enzymes in milk and dairy products: Quality aspects. **Trends in Food Science & Technology**, v.8, n.2, p.35-41, 1997.

TEH, K.H.; FLINT, S.; Palmer, J. et al. Proteolysis produced within biofilms of bacterial isolates from raw milk tankers. **International Journal of Food Microbiology**, v.157, n.1, p.28-34, 2012.

Recebido para publicação em 21/09/2014 e aprovado em 22/12/2014.