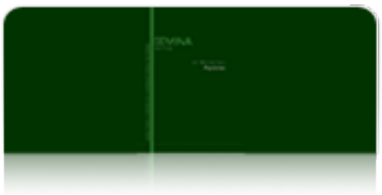

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Proteolytic behavior of isolated *Pseudomonas* spp. from refrigerated raw milk in different concentrations and storage temperatures

Comportamento proteolítico de *Pseudomonas* spp. isoladas de leite cru refrigerado em diferentes concentrações e temperaturas de estocagem

Ana Cristina Pinesso Ribeiro[1]; Samera Rafaela Bruzaroski[2]; Flavia de Almeida Bergonse Pereira[1]; Fernanda Gonzales Paião[3]; Regina Célia Poli-Frederico[4]; Joice Sifuentes dos Santos[5]; Elsa Helena Walter de Santana[5*]

Abstract

The objective of the work was to evaluate the multiplication capacity and proteolytic activity of different *Pseudomonas* spp. cell counts inoculated in milk and storaged under different temperature. Strains isolated from refrigerated raw milk (RRM) were confirmed at genus level by Polymerase Chain Reaction (PCR). The *Pseudomonas* spp. was cultured in cephalothin-sodium fusidate-cetrimide (CFC) agar-base (30°C for 48 h) until it reached 2 log and 6 log CFU mL^{-1}. Three of eight strains confirmed as *Pseudomonas* spp. were inoculated in sterile reconstituted whole milk powder and incubated at 2°C, 4°C, and 8°C for 96 h. Primary proteolysis indices was determined by the Kjeldahl method. When taking into account the effect of storage time in *Pseudomonas* spp. population, it was found that the initial population (2 log CFU mL^{-1}) showed significant difference in growth rates only from 0 h to 24 h, keeping at the same levels along 96 h. When a higher initial population was incubated (6 log CFU mL^{-1}), it was not observed a significant difference for times tested. Related to the effect of storage time in proteolysis index, it was not observed a significant difference in samples inoculated with 2 and 6 log CFU mL^{-1} *Pseudomonas* spp. When we analyzed the influence of storage temperature on the bacterial multiplication, there was a significant difference in the *Pseudomonas* spp. population only between 2°C and 8°C after 96 h of milk storage with 2 log CFU/mL of initial inoculum. If we consider the temperature effect in the primary proteolysis index, there were significant differences at the inoculum of 2 log CFU mL^{-1} where the primary proteolysis at 24 h was lower at 2°C than at 8ºC. Low temperatures or short storage time had no influence on *Pseudomonas* spp. enumeration or in the primary proteolysis index when high initial contaminations are observed. At lower *Pseudomonas* spp. initial population, the smaller storage time tested influenced the population control, and linked with the reduction in the storage temperature, lower proteolysis index were observed.

Key words: Storage. Protease. Psycrotrophic. Quality.

[1] Discente, Mestrado em Ciência e Tecnologia do Leite e Derivados, Universidade Norte do Paraná, UNOPAR, Londrina, PR, Brasil. E-mail: ana.pinesso@gmail.com

[2] Discente, curso de Medicina Veterinária, Campus Piza, UNOPAR, Londrina, PR, Brasil. E-mail: samera.rafaela@hotmail.com

[3] Pesquisadora, Mestrado em Ciência e Tecnologia do Leite e Derivados, UNOPAR, Londrina, PR, Brasil. E-mail: fergonzalesp@hotmail.com

[4] Profª, Mestrado em Ciências da Reabilitação, UNOPAR, Londrina, PR, Brasil. E-mail: reginafrederico@yahoo.com.br

[5] Profªs, Mestrado em Ciência e Tecnologia do Leite e Derivados, UNOPAR, Londrina, PR, Brasil. E-mail: joice.sifuentes@gmail.com; elsahws@hotmail.com

[*] Author for correspondence

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Resumo

O objetivo deste trabalho foi avaliar a capacidade de multiplicação e a atividade proteolítica de diferentes populações de *Pseudomonas* spp. inoculadas em leite e estocadas em diferentes temperaturas. Cepas isoladas de leite cru refrigerado (LCR) tiveram o gênero confirmado pela Reação em Cadeia da Polimerase (PCR). *Pseudomonas* spp. foram isoladas em ágar pseudomonas adicionado de cephalothin-sodium fusidate-cetrimide (30°C por 48 h) até atingir as populações de 2 log e 6 log UFC mL^{-1}. Três de oito cepas confirmadas como *Pseudomonas* spp. foram inoculadas em leite em pó integral reconstituído esterilizado e incubadas a 2˚C, 4˚C e 8˚C por 96 h. O índice de proteólise primária foi determinado pelo método de Kjeldahl. Considerando o efeito do tempo de estocagem na multiplicação de *Pseudomonas* spp., observou-se que nas baixas populações iniciais (2 log CFU mL^{-1}) houve diferença significativa na multiplicação bacteriana apenas de 0 h para 24 h, mantendo-se estável ao longo das 96 horas de estocagem. Quando uma população inicial mais alta (6 log UFC mL^{-1}) foi estocada, não observou-se diferença significativa entre o tempo zero e os demais testados. Em relação ao efeito do tempo de estocagem no índice de proteólise, não foi encontrada diferença significativa neste índice, nas amostras com inóculo inicial de 2 e 6 log UFC mL^{-1} de *Pseudomonas* spp. Quando foi analisado o efeito da temperatura de estocagem na multiplicação de *Pseudomonas* spp., houve diferença significativa apenas entre 2°C e 8°C depois de 96 h de estocagem com inóculo inicial de 2 log UFC mL^{-1}. Se for considerado o efeito da temperatura sobre a proteólise, houve diferença no inóculo 2 log UFC mL^{-1} onde a proteólise primária com 24 horas foi menor a 2°C que a 8ºC. Baixas temperaturas ou menor tempo de estocagem não tiveram influência na contagem de *Pseudomonas* spp. ou no índice de proteólise primária quando as amostras continham maior contaminação. Em baixas contagens iniciais de *Pseudomonas* spp., o menor tempo de estocagem testado influenciou no controle populacional, e associado a redução na temperatura de estocagem, foram observados menores índices de proteólise.

Palavras-chave: Estocagem. Protease. Psicrotrófico. Qualidade.

The storage of refrigerated raw milk (RRM) for long periods is problematic for maintaining its quality as well as that of its derivatives. The spoilage of milk occurs because of the growth of psychrotrophic microorganisms that affects the milk quality (SILVA et al., 2011). The psychrotrophic bacteria found in milk are mostly Gram-negative, originated from the environment and milking equipments (FAGUNDES et al., 2006; SILVA et al., 2011). This increased microbial population is due to poor hygienic conditions during milking (SILVA et al., 2011). The psychrotrophic bacteria, from a minimum population of 10^6–10^7 (6-7 log) colony forming units (CFU) mL^{-1} produce proteases and/or thermoresistant extracellular lipases which reduces the quality as well as the shelf life of the milk as evidenced by changes in the taste, smell and appearance, loss of consistency and gelling (ARCURI et al., 2008).

Pseudomonas spp. are Gram-negative psychrotrophic bacteria commonly isolated from RRM (ARCURI et al., 2008; FAGUNDES et al., 2006; NEUBECK et al., 2015; PINTO et al., 2006; XIN et al., 2017) because of its higher multiplication capacity in a refrigerated environment (KUMARESAN et al., 2007).

The production of enzymes such as proteases, lipases, and phospholipases by various *Pseudomonas* spp. are affected by temperature, oxygen availability, environmental factors, pH, and substrate concentration, as well as the growth stage of the microorganism (NUÑEZ; NUÑEZ, 1983). Enzyme production by *Pseudomonas* spp. strains occurs mainly at the end of the log phase of cell growth and at sub-optimal temperatures (MAHIEU, 1991). Hence, our aim was to assess the multiplication capacity and proteolytic activity of different *Pseudomonas* spp. cell counts isolated from RRM under different storage temperature for 96 h.

Pseudomonas spp. studied in this experiment were isolated under aseptic conditions from raw milk samples stored in cooling tanks from five dairy

farms, in Parana State, Brazil (ALMEIDA et al., 2017). Samples were kept in styrofoam box with reusable ice packs until analysis. The *Pseudomonas* spp. strains were isolated from cephalothin-sodium fusidate-cetrimide (CFC) agar-base (Himedia, Mumbai, India) by incubation at 30ºC for 48 h (FAGUNDES et al., 2006). The isolated strains were later stored at –20ºC in Brain Heart Infusion (BHI) broth (Himedia, Mumbai, India) containing 40% glycerol.

For confirming the genus *Pseudomonas* spp., ten strains were selected and identified by polymerase chain reaction (PCR) using forward PA-GS-F (GACGGGTGAGTAATGCCTA) and reverse PA-GS-R (CACTGGTGTTCCTTCCTATA) primers which amplified the specific genus region on 16S rRNA (618 bp) and amplification conditions described by Spilker et al. (2004).

Bacterial genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, USA) following the manufacturer´s instructions. The isolated DNA was stored at –80ºC. The extracted genetic material was subjected to PCR using the protocol described by Spilker et al. (2004) with modifications. Reactions were composed of a total of 25 µL consisting of 15.8 µL water, 2 µL of DNA (100 ng/uL), 2.5 µL of 10x buffer, 1.5 µL $MgCl_2$ (25 mM), 2.0 µL dNTPs (2.5 mM), 0.5 µL (0.5 rM) of each oligonucleotide (PA-GS-F and PA-GS-R) as initiators, and 1.0 U (0.2 µL) of GoTaq® DNA polymerase (Invitrogen, CA, USA).

The PCR was carried out in a thermocycler (Veriti® 96-Well, Applied Biosystems™, USA) using the following cycling parameters: 95°C for 2 min, 39 cycles of 95°C for 40 s, 54°C for 30 s, 72°C for 45 s, and one final cycle of 72°C for 7 min. Ultrapure water was used as the negative control and DNA from the strain *P. aeruginosa* (ATCC 27853), *P. fluorescens* (ATCC 13525) and *P. putida* (ATCC 31483) were used as positive controls. The PCR product was subjected to agarose gel (1%) electrophoresis, and the gel was stained with Sybr Safe (Invitrogen, CA, USA), followed by photo-documentation under ultraviolet light.

From eighth strains confirmed to be *Pseudomonas* spp., three were used for the experiment to verify if they are proteolytic and the effect of storage time and temperature on their behavior. Strains of *Pseudomonas* spp. stored at –20ºC were inoculated in 200 mL of 12% reconstituted whole milk (Nestlê, São Paulo, Brazil) (SANTOS et al., 2010) and incubated at 21ºC for 48 h. Subsequently, the number of viable cells of *Pseudomonas* spp. was determined by surface plating on CFC agar-base, at 30ºC for 48 h (FAGUNDES et al., 2006). After reaching the required bacterial cell count, decimal dilutions were made in 0.85% saline solution to obtain final concentrations of 2 log (10^2) and 6 log (10^6) CFU mL^{-1}. Each selected dilution was immediately used to set up cultures.

Aliquots of 400 mL of reconstituted whole milk were sterilized at 121°C for 15 min, mixed with 4 mL of the *Pseudomonas* inocula previously prepared (2 and 6 log CFU mL^{-1}) and incubated at 2ºC, 4ºC, and 8ºC for 96 h. *Pseudomonas* spp. enumeration and determination of proteolysis indices were carried out every 24 h in duplicate and triplicate, respectively, and time zero was considered as negative control. Three experiments were carried out with the three strains confirmed to be *Pseudomonas* spp.

The two final concentrations used in this research were selected in order to simulate, first, a lower *Pseudomonas* spp. population (2 log CFU mL^{-1}) and second, a milk with a minimum population of 6 log CFU mL^{-1} to produce thermoresistant extracellular enzymes (ARCURI et al., 2008; MAHIEU, 1991).

The temperature of 4ºC was selected to simulate Brazilian's regulations temperature in raw milk at dairy farms (BRASIL, 2011). Milk storage at 2 ºC and 8ºC were related (ALMEIDA et al., 2017) as usual in RRM and some authors had detected proteolytic activity at these temperatures, even in small psychrotrophic counts (HARYANI et al., 2003; WIKING et al., 2002).

The milk samples inoculated with *Pseudomonas* spp. were diluted in 0.85% saline solution and cultured by surface plating on CFC agar-base at 30ºC for 48 h (FAGUNDES et al., 2006).

To evaluate proteolysis, the protein fractions were determined by the Kjeldahl method (AOAC, 2000), estimating the total nitrogen content (TN) and non-casein nitrogen (NCN) (VIANNA; GIGANTE, 2010). The primary proteolysis index was determined from the NCN/TN ratio.

The results obtained were subjected to Tukey's test at 5% significance ($p < 0.05$) with the support of the software Statistica 13.0 (STATSOFT, OK, USA).

When taking into account the effect of storage time (96 h) in *Pseudomonas* spp. counting, it was found that the initial population of 2 log CFU mL^{-1}, when stored at 2°C, 4°C, and 8°C, showed significant difference in growth rates only from 0 h to 24 h ($p < 0.05$) (Table 1), keeping at the same levels along 96 h ($p > 0.05$). This result indicates that there was *Pseudomonas* spp. multiplication in the first 24 h, emphasizing the importance in reducing the storage time to control *Pseudomonas* spp. growth in RRM, contributing to lower protease sinthesis and better milk and dairy products quality. Santos et al. (2010) reported that the storage of RRM for periods longer than 48 h is detrimental for milk quality and its derivative products, due to the increase in psychrotrophic microorganism count, attesting the fact that longer the storage time, the higher the bacterial count.

Table 1. *Pseudomonas* spp. counting* (log CFU mL^{-1}) in sterile whole milk following initial inoculation of 2 and 6 log CFU mL^{-1} incubated at 2°C, 4°C and 8°C for 96 h.

Initial population (CFU mL^{-1})	TºC	Time (hours)				
		0	24	48	72	96
2 log	2	2.3^{b}	5.1^{a}	5.2^{a}	5.5^{a}	5.5$^{a, B}$
	4	2.3^{b}	4.7^{a}	5.4^{a}	5.7^{a}	5.8$^{a, A,B}$
	8	2.3^{b}	6.2^{a}	6.4^{a}	6.4^{a}	7.0$^{a, A}$
6 log	2	6.6	6.6	7.1	7.2	7.1
	4	6.6	8.2	7.8	7.6	7.9
	6	6.6	7.7	8.1	8.5	9.5

*average of three experiments

^{A,B} Capital superscripted letters in the same column indicate significant differences ($p < 0.05$) among the incubation temperature, at the same time point, and the same inoculum.

^{a,b,c} Small superscripted letters in the same line indicate significant differences ($p < 0.05$) among the different incubation times, at the same temperature, and the same inoculum.

When a higher initial population was incubated (6 log CFU mL^{-1}), it was not observed a significant difference ($p > 0.05$) between time zero and the others times tested (Table 1), stressing the relevancy of low initial *Pseudomonas* spp. populations in milk prior to the refrigeration. Researches showed high counts of *Pseudomonas* spp. in refrigerated raw milk, with populations between 4 and 6 log CFU mL^{-1} (ALMEIDA et al., 2017; FAGUNDES et al., 2006). Related to the effect of storage time in proteolysis index, it was not observed a significant difference ($p > 0.05$) in samples inoculated with 2 or 6 log CFU mL^{-1} *Pseudomonas* spp. (Table 2). Studies assessing the time and refrigeration temperature effects on the growth of psychrotrophic bacteria in milk, indicate that raw milk with high counts of psychrotrophic and proteolytic psychrotrophic bacteria do not necessarily have more elevated proteolytic activity compared to samples having lower counts (SANTOS et al., 2010).

Table 2. Primary proteolysis (%)* of sterile whole milk inoculated with *Pseudomonas* spp. (2 and 6 log CFU mL^{-1}) incubated at 2°C, 4°C and 8°C for 96 h.

Initial population (CFU mL^{-1})	TºC	Time (hours)				
		0	24	48	72	96
2 log	2	4.44	7.65[B]	7.67	8.25	5.41
	4	6.10	9.71[A,B]	7.77	8.70	6.29
	8	7.83	10.64[A]	10.21	9.58	5.36
6 log	2	5.67	8.41	9.61	8.51	5.21
	4	6.27	7.08	7.21	8.94	4.20
	8	9.45	8.42	9.44	8.80	6.09

*average of three experiments

[A,B] Capital superscripted letters in the same column indicate significant differences ($p < 0.05$) among the incubation temperature, at the same time point, and the same inoculum.

When we analyzed the influence of storage temperature of each inoculum for the same period of incubation on the bacterial multiplication, there was a significant difference ($p < 0.05$) in the *Pseudomonas* spp. population only between 2°C and 8°C after 96 h of milk storage with 2 log *Pseudomonas* spp. CFU mL^{-1} inocula. Thus, in this study, the storage at 2ºC was enough to control bacterial growth only at the lower inoculum tested until 96 h, indicating, again, the importance of lower initial counts of *Pseudomonas* spp. Pinto et al. (2006) evaluated milk samples inoculated with 4 log CFU mL^{-1} of *P. fluorescens* at 2, 4, 7 and 10 ºC and found that the temperatures of 2 ºC and 4 ºC were effectives to control bacterial growth after 24 h of incubation. After 48 h, a higher growth rate was observed in samples stored at higher temperatures. We asserted that even in refrigeration temperatures proposed by Brazilian legislation (BRASIL, 2011), a loss of quality in raw milk will occur if the initial contamination is not effectively controlled.

If we consider the temperature effect within the same time and inoculum, there were significant differences ($p < 0.05$) in the primary proteolysis index at the inoculum of 2 log CFU mL^{-1} of *Pseudomonas* spp., where the primary proteolysis at 24 h was lower at 2°C than at 8ºC. Thus, lower refrigeration temperatures contribute to lower proteolysis index only until 24 h of storage, when there is a small initial population of *Pseudomonas* spp., highlighting, once more, the importance of minimal storage time to keep the milk and dairy products quality. Kumaresan et al. (2007), comparing psychrotrophic growth in different incubation temperatures, observed that milk storage at 2 ºC resulted in lower bacterial growth and lower proteolytic and lipolytic activity.

At the primary proteolysis, called Extension Index, micelles of casein are cleaved into peptides, thereby solubilizing the casein. The greater the primary proteolysis, the more soluble peptides will be released from the casein, being κ-casein and glycomacropeptides the most important. The fraction κ-casein located at the surface of the casein micelle is preferentially hydrolyzed by psychrotrophic bacterial proteases and this hydrolysis causes the development of bitter taste and increases the viscosity, with eventual gel formation in ultra-high temperature milk, when subjected to long storage period (FOX; MCSWEENEY, 1998).

Thus, low temperatures or short storage time had no influence on *Pseudomonas* spp. enumeration or in the primary proteolysis index when high initial contaminations are observed. At lower *Pseudomonas* spp. initial population, the smaller storage time tested influenced the population control, and linked with the reduction in the storage temperature, lower proteolysis index were observed.

References

ALMEIDA, K. M.; BRUZAROSKI, S. R.; ZANOL, D.; MELO, M.; SANTOS, J. S.; ARAGON ALEGRO, L. C.; SANTANA, E. H. W. *Pseudomonas* spp. and *P. fluorescens*: population in refrigerated raw milk. *Ciência Rural*, Santa Maria, v. 47, n. 1, p. 1-5, 2017.

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS – AOAC. Official methods of analysis of AOAC International. 2000. 17th ed. Gaithersburg, Maryland: AOAC International.

ARCURI, E. F.; SILVA, P. D. L.; BRITO, M. A. V. P.; BRITO, J. R. F.; LANGE, C. C.; MAGALHÃES, M. M. A. Counting, isolation and characterization of psychrotrophic bacteria from refrigerated raw milk. *Ciência Rural*, Santa Maria, v. 38, n. 8, p. 2250-2255, 2008.

BRASIL. Ministério da Agricultura, Pecuária e Abastecimento: Instrução Normativa 62, de 29 de dezembro de 2011. Aprova o regulamento técnico de produção, identidade e qualidade do leite tipo A, o regulamento técnico de identidade e qualidade de leite cru refrigerado, o regulamento técnico de identidade e qualidade de leite pasteurizado e o regulamento técnico da coleta de leite cru refrigerado e seu transporte a granel. *Diário Oficial [da República Federativa do Brasil],* Brasília, 30 dez. 2011. Seção 1, p. 6.

FAGUNDES, C. M.; FISCHER, V.; SILVA, W. P.; CARBONERA, N.; ARAÚJO, M. R. Presença de Pseudomonas spp. em função de diferentes etapas da ordenha com distintos manejos higiênicos e no leite refrigerado. *Ciência Rural*, Santa Maria, v. 36, n. 2, p. 568-572, 2006.

FOX, P. F.; McSWEENEY, P. L. H. *Dairy chemistry and biochemistry*. Press: Tullamore, Ireland, 1998. 478 p.

HARYANI, S.; DATTA, N.; ELLIOTT, A. J.; DEETH, H. C. Production of proteinases by psychrotrophic bacteria in raw milk stored at low temperature. *Australian Journal of Dairy Technology*, North Melbourne, v. 58, n. 1, p. 15-20, 2003.

KUMARESAN, G.; ANNALVILLI, R.; SIVAKUMAR, K. Psychrotrophic spoilage of raw milk at different temperatures of storage. *Journal of Applied Sciences Research***,** Amman-Jordan, v. 3, n. 1, p. 1383-1387, 2007.

MAHIEU, H. Modificaciones de la leche después de su recogida. In: LUQUET, F. M. *Leche y productos lácteos. La leche de la mama a la lechería*. Zaragoza: Acribia, p. 181-22,1991.

NEUBECK, M.; BAUR, C.; KREWINKEL, M.; STOECKEL, M.; KRANZ, B.; STRESSLER, T. FISCHER, L.; HINRICHS, J.; SCHERER, S.; WENNING, M. Biodiversity of refrigerated raw milk microbiota and their enzymatic spoilage potential. *International Journal of Food Microbiology*, Torino, v. 211, p. 57-65, 2015.

NUÑEZ, M.; NUÑEZ, J. A. Proteasas de psicrotrofos Gram negativos. Efectos sobre la leche y los productos lácteos. *Revista Española de Lecheria*, Madrid, v. 130, n. 1, p. 251-260,1983.

PINTO, C. L. O.; MARTINS, M. L.; VANETTI; DANTAS, M. C. Qualidade microbiológica de leite cru refrigerado e isolamento de bactérias psicrotróficas proteolíticas. *Food Science and Technology*, Campinas, v. 26, n. 3, p. 645-651, 2006.

SANTOS, P. A.; SILVA, M. A. P.; MOREIRA, N, G.; BARROS, C. J.; OLIVEIRA, A. N.; NICOLAU, E. S. Evolução da proteólise do leite inoculado *in vitro* com *Pseudomonas fluorescens. Boletim do Centro de Processamento de Alimentos*, Curitiba, v. 28, n. 2, p. 313-320, 2010.

SILVA, L. C. C.; BELOTI, V.; TAMANINI, R.; OVIDIO, L.; MATTOS, M. R.; ARRUDA, A. M. C. T.; PIRES, E. M. F. Rastreamento de fontes da contaminação microbiológica do leite cru durante a ordenha em propriedades leiteiras do Agreste Pernambucano. *Semina: Ciências Agrárias*, Londrina, v. 32, n. 1, p. 267-276, 2011.

SPILKER, T.; COENYE, T.; VANDAME, P.; LIPUMA, J. J. PCR-Based assay for differentiation of Pseudomonas aeruginosa from other Pseudomonas species recovered from cystic fribrosis patients. *Journal of Clinical Microbiology,* Washington D.C., v. 42, n. 5, p. 2074-2079, 2004.

VIANNA, P. C. B.; GIGANTE, M. Physical chemical quality of the refrigerated raw milk added with carbon dioxide. *Revista do Instituto Laticínio Cândido Tostes*, Juiz de Fora, v. 375, n. 65, p. 51-59, 2010.

WIKING, L.; FRØST, M. B.; LARSEN, L. B.; NIELSEN, J. H. Effects of storage condition on lipolysis, proteolysis and sensory attribute in high quality raw milk. *Milchwissenschaft*, Kempten, v. 57, n. 4, p. 190-194, 2002.

XIN, L.; ZHAOXU, M.; ZHANG, L.; CUI, Y.; HAN, X.; YI, H. The diversity and proteolytic properties of psychrotrophic bacteria in raw cows' milk from North China. *International Dairy Journal*, Edmonton, v.66, March, p. 34-41, 2017.