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Presence of *aprX* gene in *Pseudomonas* spp. from refrigerated raw milk and their proteolytic ability

Presença do gene *apr*X em *Pseudomonas* spp. isolados de leite cru refrigerado e sua capacidade proteolítica

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Highlights:
P. fluorescens and *P. putida* represented 62.4% of *Pseudomonas* spp.
The mean probability for occurrence the *aprX* gene in *Pseudomonas* species was 72%.
The *aprX* gene was not associated with the low or high proteolytic potential.

Abstract

This study aimed to determine the frequency of *Pseudomonas fluorescens*, *P. putida*, and *P. aeruginosa* in refrigerated raw milk; their proteolytic potential; and your association with the *aprX* gene. Of the 173 isolates confirmed as belonging to *Pseudomonas* spp., 37% were *P. fluorescens*, 25.4% *P. putida*, and none belongs to *P. aeruginosa*. The *aprX* gene was distributed proportionally between *P. putida* (68%) and *P. fluorescens* (75%), but it was not associated with low or high proteolytic potential in both species. *P. putida* (16) and *P. fluorescens* (14) isolates with no *aprX* gene identified also had proteolytic potential. Considering the synthesis of proteases other than AprX by the isolates under study, we concluded that *P. fluorescens* and *P. putida* represented 62.4% of the *Pseudomonas* genus, with high probability of having the *aprX* gene and proteolytic potential. However, there was no association between the deteriorating potential with the presence of the *aprX* gene.
Key words: Psychrotrophic. Enzyme. Quality. Proteolysis.

Resumo

O objetivo do estudo foi verificar a frequência, em leite cru refrigerado de *Pseudomonas fluorescens*, *P. putida* e *P. aeruginosa*, o potencial proteolítico dos isolados e sua associação com a presença do gene *apr*X. Dos 173 isolados confirmados como pertencente ao gênero *Pseudomonas* spp, 37% foram *P. fluorescens,* 25,4% *P. putida* e nenhum pertencia à espécie *P. aeruginosa*. A prevalência do gene *apr*X se distribuiu proporcionalmente entre *P.putida* (68%) e *P.fluorescens* (75%) porém não foi associada ao baixo ou alto potencial proteolítico nas duas espécies avaliadas. *P. putida* (16) e *P. fluorescens* (14) que não tiveram o gene *apr*X identificado também expressaram potencial proteolítico, considerando-se

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a síntese de outras proteases além da AprX pelos isolados estudados. Concluiu-se que *Pseudomonas fluorescens* e *P. putida* representaram 62,4 % do gênero *Pseudomonas* ssp., com alta probabilidade da presença do gene *apr*X e potencial proteolítico, porém sem associação da expressão do potencial deteriorante com a presença do gene *apr*X.
Palavras-chave: Psicrotrófico. Enzima. Qualidade. Proteólise.

Pseudomonas are considered the predominant psychrotrophic genus in cold raw milk producing heat resistant enzymes that cause off-flavour mainly in dairy products (Xin et al., 2017). *Pseudomonas* spp. have high genetic diversity, metabolic versatility and an efficient cold adaptation linked to the possession of elevated levels of unsaturated lipids in their membranes. The species most commonly isolated from milk, milking environments, and industrial plants are *P. fluorescens*, *P. aeruginosa*, and *P. putida* (Dogan & Boor, 2003; Decimo, Morandi, Silvetti, & Brasca, 2014).

Pseudomonas spp. are known for their production of alkaline metalloprotease (AprX) in the cold chain of raw milk. These enzyme is encoded by *aprX* gene which primarily catalyze the cleavage of internal bonds of peptides. These enzymes hydrolyze milk protein, mainly caseins that are very susceptible to proteolysis because they contain random nonhelical strucutres (Martins, Araújo, Mantovani, Moraes, & Vanetti, 2005).

This study aimed to determine the frequency of *P. fluorescens*, *P. putida*, and *P. aeruginosa* in refrigerated raw milk, as well as the proteolytic potential of the isolates and their association with the *aprX* gene.

Milk samples (10) were collected from refrigerated raw milk that was sent for processing. After 48 h of refrigeration in bulk tanks in the dairy farms, *Pseudomonas* spp. were isolated from CFC-supplemented (cefaloridine, fusidic acid, cetrimide) *Pseudomonas* agar base (Himedia, Mumbai, India) at 30°C for 48 h (Fagundes, Fischer, Silva, Carbonera, & Araujo, 2006; Almeida et al., 2007).

An agar plate containing 25 to 250 colonies of *Pseudomonas* spp. was selected for each sample, and all the isolated strains were evaluated for proteolytic potential. To determine this, skim milk agar(10%) was incubated at 21°C (Frank & Yousef, 2004). After 72 hours, clear halos, depicting proteolytic activity by psychrotrophic micro-organisms, were measured. In these study, isolates with halos ≤ 2 cm and > 2 cm wide, were classified as possessing low and high-potential proteolytic activity, respectively. The cutoff values for the proteolytic activity (high or low) were based on the mean and standard deviation of our own data, always avoiding less than 40 observations for each category

After proteolytic evaluation 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 80°C and the extracted genetic material was utilized in polymerase chain reaction (PCR). For *Pseudomonas* spp identification were used primers and reaction conditions described by Spilker, Coenye, Vandame and Lipuma (2004) (Table 1). The species *P. fluorescens*, *P.putida* and *P. aeruginosa* were identified with PCRs protocols described by Scarpellini, Franzetti and Galli (2004), Yamamoto and Harayama (1995) and Spilker et al. (2004), respectively (Table 1).

Amplification of the *aprX* gene from the *Pseudomonas* spp. isolates was performed according to Bach, Hartmann, Schloter and Munch (2001). Ultra-pure water was used as negative control and the *P. fluorescens* (ATCC 13525), *P. aeruginosa* (ATCC 27853) and *P. putida* (ATCC 31483) strains were used as positive control (Table 1).

The occurrence of the *aprX* gene among the *Pseudomonas* species was assessed by the univariate Chi-square test (X^2). The test was also used to assess the association between the presence of the gene and the potential for proteolysis.

Table 1
Primers used for identification of *Pseudomonas* spp and *aprX* gene

Gene	Primers (5'-3')	Size (pb)	Reference
16S rRNA *Pseudomonas* genus-specific	PA-GS-F (5′-GACGGGTGAGTAATGCCTA-3′) PA-GS-R (5′-CACTGGTGTTCCTTCCTATA-3′)	618	Spilker et al., 2004
P. fluorescens	16 SPSEfluF (5'-TGCATTCAAAACTGACTG-3') 16SPSER (5'-AATCACACCGTGGTAACCG-3')	800	Scarpellini et al., 2004
P. putida	P734 (5'-CAA CTCGGGCGTTGGCATTCTGCT-3') P1455r (5'-CAAGATCGCCTGGGTACGACGGTT-3')	744	Yamamoto and Harayama, 1995
P. aeruginosa	PA-SS-F (5-'GGGGGATCTTCGGACCTCA-3') PA-SS-R (5-'TCCTTAGAGTGCCCACCCG-3')	956	Spilker et al., 2004
aprX	FP*apr*l (5' - TAYGGBTTCAAYTCCAAYAC-3') RFaprII (5' - VGCGATSGAMACRTTRCC-3')	194	Bach et al., 2001

Of the 173 isolates confirmed as belonging to the genus *Pseudomonas* spp., 37% (64) were identified as *P. fluorescens* and 25.4% (44) as *P. putida*. No *P. aeruginosa* isolate was identified. *P. fluorescens* originates from water and soil, and according to the literature, it is the most frequent species in refrigerated raw milk and milking environments (Decimo et al., 2014; Oliveira, Favarin, Luchese, & MClintosh, 2015; Meng et al., 2017). *P. putida* is often isolated from water and soil and can multiply fast (Timmis, 2002). Its presence in milk has been described (Meng et al., 2017; Decimo et al., 2014), but at lower frequencies than those found in our study.

With regard to the presence of the *aprX* gene, the mean probability for occurrence in *Pseudomonas* species was 72%, distributed proportionally ($X^2 = 0.60$, $p = 0.44$) between *P. putida* (68%) and *P. fluorescens* (75%) (Table 2). The presence of the *aprX* gene was characterized in several *Pseudomonas* spp., particularly *P. fluorescens* (Martins et al., 2005; Meng et al., 2017). The presence of gene *aprX* it is associated with sensorial defects throughout the shelf-life of dairy products, such as gelatinization in UHT milk, reduction in cheese yield, consistency and texture and sensory defects such as bitter taste (Oliveira et al., 2015). Proteinases preferentially hydrolyze casein κ, then casein β, then casein αS1 and are usually thermostable, keeping their activity even after pasteurization and ultra-high temperature (UHT) treatments (Decimo et al., 2014).

In our study, the *aprX* gene was not associated with the low or high proteolytic potential in both species. Therefore, regardless of the intensity of proteolysis of the P. *fluorescens* and *P. putida* isolates, the microbiota of the refrigerated raw milk under study was composed of *Pseudomona*s species mostly having the *aprX* gene and expressing proteolytic potential, which can limits the shelf-life and quality of dairy products.

The results showed that all *P. putida* (16) and *P. fluorescens* (14) isolates without the *aprX* gene expressed proteolytic potential, with 71% of *P. putida* strains and 62% of *P. fluorescens* strains showing halos greater than 2 cm. Therefore, it must be associate proteolytic potential with the production of other proteases. *P. fluorescens* frequently produces, at least, one extracellular proteinase with molecular weight ranging between 45 and 56 kDa, which corresponds to AprX (Decimo, Morandi, Machado, Bagliniere, & Vanetti, 2018). Stuknytë et al. (2016) studied the thermostable proteolytic activity of *P. fluorescens* PS19 isolated from raw bovine milk and described two other thermostable proteolytic bands on a zymogram. These proteases possessed molecular masses of approximately 15 and 25 kDa,

where the former was identified as a fragment of the AprX protease and the 25-kDa protease was not homologous to any known *Pseudomonas* spp. protein.

Table 2
Prevalence* of the *aprX* gene in the *Pseudomonas putida* and *Pseudomonas fluorescens* species and their proteolytic potential (high and low)(1)

Species	Absolute frequency	Prevalence of *aprX* gene (%)	X^2	*p*
P. putida	30	68.8		
P. fluorescens	48	75.00	0.60	0.44
Proteolytic potential				
P. putida				
High	20	66,67		
Low	7	63,64		
None	3	100,00	1.54	0.46
Proteolytic potential				
P. fluorescens				
High	34	77,27		
Low	12	66,67		
None	2	100,00	1.45	0.48

*Univariate chi-square test (X^2). (1)After 21 °C/72 hs in 10% milk agar, clear halos ≤ 2 cm and >2 cm wide were classified to signify low and high proteolytic potential, respectively.

Therefore, *P. fluorescens* and *P. putida* represented 62.4% of *Pseudomonas* spp., with high probability of having the *aprX* gene and proteolytic potential. However, there was no association between the expression of the deteriorating potential with the presence of the *aprX* gene, indicating that these micro-organisms may be genetically prepared for the synthesis of other proteases if there is metabolic demand.

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