Journal of Dairy Research

cambridge.org/dar

Pseudomonas fluorescens and *Pseudomonas putida* from refrigerated raw milk: genetic diversity and lipoproteolytic activity

Rosana De Longhi[1], Selma de Souza Correia[2], Samera Rafaela Bruzaroski[1], Regina Celia Poli-Frederico[3], Rafael Fagnani[4] and Elsa Helena Walter de Santana[1,2]

[1]Universidade Pitágoras Unopar (UNOPAR), Mestrado em Ciência e Tecnologia de Leite e Derivados, Londrina, Paraná, Brazil; [2]UNOPAR, Mestrado em Saúde e Produção Animal, Arapongas, Paraná, Brazil; [3]UNOPAR, Mestrado e Doutorado em Ciência da Reabilitação, Londrina, Paraná, Brazil and [4]Universidade Estadual de Londrina, Londrina, Paraná, Brazil

Research Article

Cite this article: Longhi RD, Correia SdeS, Bruzaroski SR, Poli-Frederico RC, Fagnani R and Santana EHW de (2022). *Pseudomonas fluorescens* and *Pseudomonas putida* from refrigerated raw milk: genetic diversity and lipoproteolytic activity. *Journal of Dairy Research* **89**, 86–89. https://doi.org/10.1017/S0022029922000048

Received: 31 May 2021
Revised: 11 November 2021
Accepted: 11 November 2021
First published online: 11 February 2022

Keywords:
Enzymes; psychrotrophic; quality; Rep PCR

Author for correspondence:
Elsa Helena Walter de Santana,
Email: elsahws@hotmail.com

Abstract

In this research communication the genetic diversity of *Pseudomonas fluorescens* ($n = 67$) and *Pseudomonas putida* ($n = 44$) isolated from refrigerated raw milk from bulk tank trucks were verified. The relationship between the genetic profile of the isolates and their lipoproteolytic potential was evaluated using skim milk agar and tributyrin agar (21°C/72 h). The lipoproteolytic potential (low or high), evaluated by the diameter of the halos (cm), was correlated with the number of milk producing properties that contributed to each sample (one sample = one bulk tank truck; 8–80 producers/sample) and the distance between the dairy properties and the processing plant (21–370 km). *P. fluorescens* was confirmed in all samples, while *P. putida* in 60% samples. For both species, two clusters (I and II) were observed, and the first one showed lower genotypic diversity and the presence of isolates with 100% similarity. *P. fluorescens* isolates presenting at least 70% similarity were 83.9% in Cluster I ($n = 31$) and 44.4% in Cluster II. In both clusters (I and II) observed in the *P. fluorescens* dendrogram, the occurrence of high proteolytic and lipolytic potential were equivalent. The higher the number of farms per milk sample, the greater the lipoproteolytic intensity of *P. fluorescens* isolates. In relation to *P. putida* isolates, 74% presented at least 50% similarity in Cluster I ($n = 27$) and only 35% in Cluster II ($n = 17$). The occurrence of high proteolysis linked to *P. putida* was proportional between both Clusters, but the occurrence of high lipolysis was greater in Cluster II. No significant association was detected between *P. putida* isolates and the variables studied. The results indicate the circulation of *P. putida* and *P. fluorescens* with 100% similarity in different milk producing regions. The level of genetic diversity was related only to the lipolytic capacity of *P. putida*.

The demand for high quality dairy products has stimulated research regarding the diversity and spoilage potential of psychrotrophic bacteria. *P. fluorescens* and *P. putida* are important psychrotrophic microorganisms that produce thermostable hydrolytic enzymes that mainly degrade milk proteins and lipids, thus posing a major problem for the dairy industry (Decimo *et al.*, 2014). According to Aguiar *et al.* (2019), the association of adequate cow milking and genetic studies evaluating *Pseudomonas* spp. strains present in the milking environment is essential to improve the quality and shelf life of dairy products.

In this work, the genetic variability of *P. fluorescens* and *P. putida* isolated from refrigerated raw milk from bulk tank trucks sent to the processing plant was determined and the relationship between the genetic profile of the species and their lipoproteolytic potential was evaluated. In addition, the lipoproteolytic potential was correlated with the number of milk producers composing a sample and the distance from the dairy property to the processing plant.

Materials and methods

Collection and source of isolates

After 48 h of refrigeration in bulk tanks in the dairy farms, milk was transported to the processing plant in trucks with isothermal tanks. Raw milk (10 samples) was collected from the tanks of the trucks containing milk from 8 to 80 dairy properties, whose distances from the processing plant ranged between 21 and 370 km.

Pseudomonas spp. was isolated from raw milk using CFC-supplemented (cefaloridine, fusidic acid, cetrimide) *Pseudomonas* agar base (Himedia, Mumbai, India) at 30°C for 48 h (Almeida *et al.*, 2017). Milk temperature was measured using a digital thermometer and ranged between 7.5 and 9.7°C, with an average of 8.5°C.

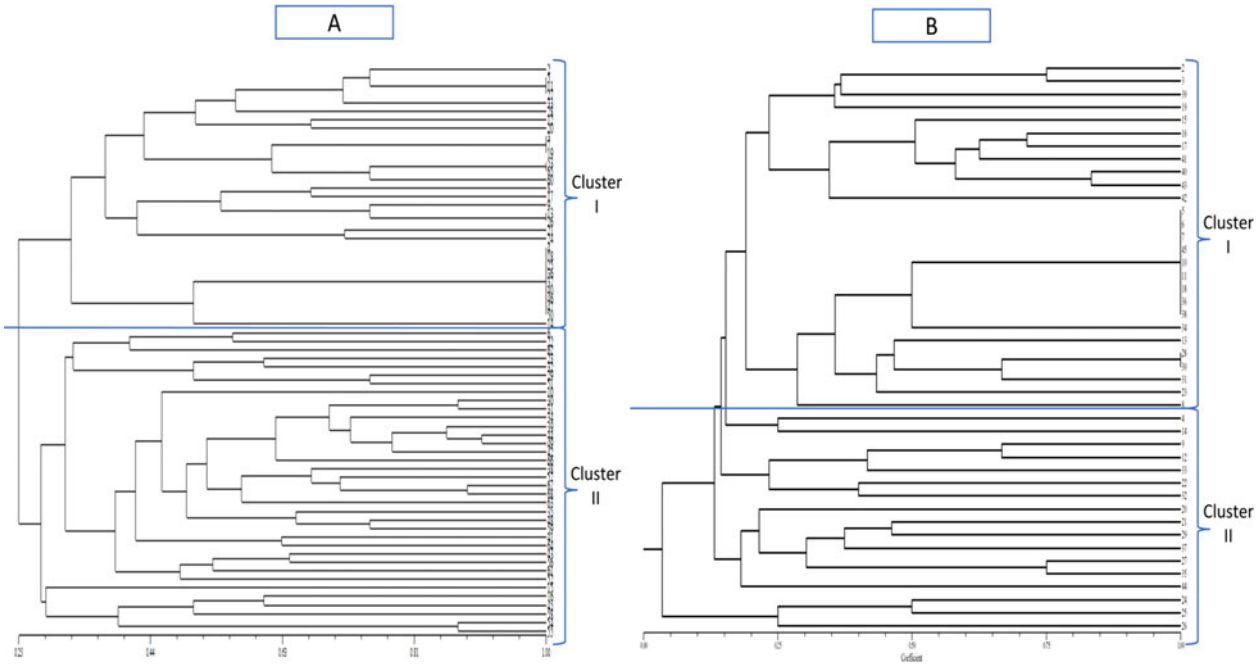

Fig. 1. Dendrogram showing genetic similarity of *P. fluorescens* (a) and *P. putida* (b). Dendrogram was constructed based on numerical analysis of REP–PCR profiles of 67 *P. fluorescens* (a) and 44 *P. putida* (b) isolates from refrigerated raw milk. (a) Cluster 1: samples A (2), B (3, 18, 46), C(4, 27, 36, 37, 65), D (9, 12, 13, 17, 19, 20, 21, 24–26), E (7, 8, 28, 53), F (5, 11, 33, 47, 60), H (40), I (14) and J (50), Cluster 2: samples A (45), B (42, 49), C (23, 35, 62), D (6, 32, 41, 63, 64, 66, 67, 68), E (10, 22, 51, 52, 54–59, 61), F(15, 16), G (29), I (34, 38) and J (30, 31, 39, 43, 44, 48). (b) Cluster 1: samples E (28), G (3, 2, 5- 8, 10, 11, 13, 15–17, 34, 36, 38, 39–43, 45), H (31) and I (18, 19, 30). Cluster 2: samples E (20–25, 27,35), F (29), G (4, 9, 12, 14, 33, 37), H (32) and J (44).

Lipoproteolytic activity

Agar plates containing 25 to 250 colonies of *Pseudomonas* spp. were selected from each sample. The isolates (111) had their proteolytic (10% milk agar) and lipolytic (tributyrin agar) potential evaluated (21°C/72 h) (Frank and Yousef, 2004) by measuring the diameter (cm) of the characteristic halos formed after 72 h. Thereafter, the isolates were grouped in categories according to their low or high lipoproteolytic potential. Isolates with clear halos measuring ≤2 cm and <1.5 cm in diameter were classified as presenting low proteolytic (PP) and lipolytic (LP) potentials, respectively. Those with halos >2 cm and ≥1.5 cm in diameter were considered of high PP and LP potentials, respectively (Aguiar *et al.*, 2019). The cutoff values for the lipoproteolytic activity (high or low) were based on the mean and standard deviation of our data, always avoiding less than 40 observations for each category.

According to the diameter of the halos (cm), the lipoproteolytic potential was correlated with the number of milk producers composing a sample and the distance from the dairy property to the processing plant using nonparametric Spearman's test with 5% of significance.

P. fluorescens *and* P. putida *molecular identification*

The extracted genetic material (Wizard Genomic DNA Purification Kit, Promega Corporation, Madison, USA) was subjected to PCR amplification for the identification of *Pseudomonas* spp. (GenBank number AY486387.1) (Spilker *et al.*, 2004). After, the species *P. fluorescens* (GenBank number CP015639.1) (Scarpellini *et al.*, 2004) and *P. putida* (GenBank number CP015876.1) (Yamamoto and Harayama, 1995) were identified and submitted to REP-PCR protocol. Ultrapure water was used as a negative control, while DNA from *P. fluorescens* (ATCC 13525) and *P. putida* (ATCC 31483) strains were used as positive controls.

Genetic similarity between the strains was assessed based on the presence or absence of a specific REP-PCR product (Louws *et al.*, 1994). Similarity matrices were constructed using the Dice coefficient. The matrices were then grouped using the UPGMA method and graphically represented as OPTICS dendrograms using the software NTSYSpc2.10 (Numerical Taxonomy and Multivariate Analysis System; Stone Brook, 1998) and dendrograms were created for *P. fluorescens* and *P. putida*. The isolates were clustered into groups (Clusters I and II), where Cluster I had isolates with 'low genetic diversity' and Cluster II 'high genetic diversity'.

The proportions between the groups (Clusters I and II) for both species were compared by Chi-square test. Fisher's exact test were used when the absolute frequency was less than 5.

Results and discussion

Sixty-seven (60.36%) *Pseudomonas* spp. isolates out of a total of 111 were *P. fluorescens*. This species was confirmed in all analyzed milk samples, with the frequency ranging from 3.57% to 100% (average 72.19%). A total of 83.9% ($n = 26$) of *P. fluorescens* isolates from Cluster I ($n = 31$), with low genetic diversity, presented at least 70% similarity, and the remainder exhibited 100% similarity. Cluster II ($n = 36$), with high genetic diversity, on the other hand, showed 44.4% ($n = 16$) of isolates with 70% similarity and none of them with 100% similarity. Interestingly, no isolates from samples G and H were observed in Clusters I and II, respectively (Fig. 1a). Thus, these results suggest the presence of a unique genetic profile of *P. fluorescens* in these two samples, which present as common characteristics related to the

https://doi.org/10.1017/S0022029922000048 Published online by Cambridge University Press

Table 1. Relative frequencies of high proteolytic and lipolytic potentials of *P. fluorescens* and *P. putida* isolated from raw milk in bulk tank truck*, clustered according to Rep-PCR analyses

	P. fluorescens		*P. putida*	
	Cluster 1	Cluster 2	Cluster 1	Cluster 2
Occurrence of high proteolytic potential (halo >2 cm)	80.6% ($n = 25$)	75% ($n = 27$)	69.2% ($n = 18$)	66.7% ($n = 12$)
	$\chi^2 = 0.30$; $P = 0.80$ $\chi^2 = 0.03$; $P = 0.86$			
Occurrence of high lipolytic potential (halo ≥1.5 cm)	22.6% ($n = 7$)	27.8% ($n = 10$)	7.7% ($n = 2$)	27.8% ($n = 10$)
	$\chi^2 = 0.24$; $P = 0.63$ Fisher, $P = 0.10$			

*The samples (10) were collected from tank trucks containing milk from 8 to 80 dairy properties.

greater proximity between the farms and the dairy plant and samples containing the milk of a smaller number of dairy properties per tank truck.

Among *P. fluorescens* isolates showing 100% similarity, four groups were identified: the first (samples C and F) and the second (samples B, D and E) groups were composed of 3 isolates each, the third group (sample D) of 2 isolates, and the fourth one contained 9 isolates from 6 different origins (samples B, C, D, F, H, and J) (Fig. 1a). So, in this research, *P. fluorescens* isolates showing a 100% similarity compose the raw milk microbiota of different dairy farms. Probably, this fact is associated with sources of contamination common to these microorganisms, such as water and milking environment (Decimo *et al.*, 2014), in addition to similar orientation of good cow milking, since the properties provide milk to the same dairy cooperative. Milkers hands, the surface of cows teats, teat cups and cooling tanks were associated with the presence of *Pseudomonas* spp. in raw milk from farms using manual or mechanical milking system, showing that, regardless of the type of system, good milking practices (GMP) are essential to avoid the contamination of raw milk (Vidal *et al.*, 2017).

Forty-four (39.64%) strains of *Pseudomonas* spp. out of a total of 111 were identified as *P. putida* and isolated from 60% (6) of milk samples, with a frequency ranging from 12.5 to 96.43%. Most isolates ($n = 20$, 74%) belonging to Cluster I ($n = 27$) (Fig. 1b) presented at least 50% similarity. The presence of isolates with 100% similarity (Fig. 1b) shows low genetic diversity among them. On the other hand, only 35% ($n = 6$) of the 17 strains belonging to Cluster II, presented 50% similarity, which indicates greater genetic diversity than Cluster I. In addition, no strains from sample I was represented in Cluster II. Two groups of *P. putida* showing 100% similarity were identified (Fig. 1b): the first, composed of 9 isolates (samples G and I) and the second, by two (samples E and I). These results demonstrate the circulation of *P. putida* 100% similar in different milk producing regions.

In both clusters (I and II) observed in the dendrogram of *P. fluorescens*, the occurrences of high proteolytic (Cluster I 80.6%; Cluster II 75%) and lipolytic (Cluster I 22.6%; Cluster II 27.8%) potentials were equivalent ($\chi^2 = 0.30$; $P = 0.80$) (Table 1). Thus, in our study, the deteriorating potential of the isolates was not linked with the level of genetic diversity of the species.

The lipoproteolytic intensity of *P. fluorescens* was associated only with the number of producers per milk sample. The greater the number of producers per sample, the greater the proteolytic capacity ($P < 0.05$; $R = 0.28$) and lipolytic ($P < 0.01$; $R = 0.44$) (Table 1) potentials of the *P. fluorescens* isolates. Enzymatic synthesis at refrigeration temperatures occurs mainly at the end of the log phase and during the stationary phase of the bacterial growth curve (Mahieu, 1991). Kumaresan *et al.* (2007) observed that milk stored at 2°C resulted in lower bacterial growth and lower proteolytic and lipolytic activities, when compared to storage at 4 and 7°C for 14 d. Since a greater number of properties per tank is usually linked to a small volume of milk produced, more studies are needed to evaluate whether variables such as temperature and storage time differ in dairy farms and how the enzymatic activity of *P. fluorescens* is affected.

The occurrence of high proteolysis by *P. putida* was proportional between Clusters I (69.2%) and II (66.7%), but the occurrence of high lipolysis was greater (Exact Fisher's test; $P = 0.10$) in Cluster II (27.8%) (Table 1). Differences in extracellular enzymatic activity of individual strains are probably associated with a particular genetic group to which they belong (Ercolini *et al.*, 2009). Although the lipolytic degradation of milk is not as intense as the proteolytic, defects resulting from the action of lipases are the first noticeable sensory changes, such as soap flavor and odor, metallic or oxidized flavor and fruity odor (Chen *et al.*, 2003). According to the data obtained for *P. putida*, no significant association was detected between the number of properties per milk sample and the distance between the dairy farm and the processing plant.

In conclusion, the results indicate the circulation of *P. putida* and *P. fluorescens* with 100% similarity in different milk producing regions. The level of genetic diversity was related only to the lipolytic capacity of *P. putida* isolates and the lipoproteolytic intensity of *P. fluorescens* was positively associated with the number of farms per bulk milk tank truck.

Acknowledgements. This work was financial supported by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior).

References

Aguiar BM, Longhi R, Poli-Frederico RC, Fagnani R and Santana EHW (2019) Lipoproteolytic capacity and potential of *Pseudomonas* spp. Isolated from cold raw milk. *Journal of Dairy Research* **86**, 467–469.

Almeida KM, Bruzaroski SR, Zanol D, Melo M, Santos JS, Aragon- Alegro LC, Botaro BG and Santana EHW (2017) Pseudomonas spp. and *P. fluorescens*: population in refrigerated raw milk.. *Ciência Rural* **47**, 2–6.

Chen L, Daniel RM and Coolbear T (2003) Detection and impact of protease and lipase activities in milk and milk powders. *International Dairy Journal* **13**, 255–275.

Decimo M, Morandi S, Silvetti T and Brasca M (2014) Characterization of Gram negative psychrotrophic bacteria isolated from Italian bulk tank milk. *Journal of Food Science* **79**, 2081–2090.

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

https://doi.org/10.1017/S0022029922000048 Published online by Cambridge University Press

Frank JF and Yousef AE (2004) Tests for groups of microorganisms. In Weir MH (ed.), *Standard Methods for the Examination of Dairy Products*. Nova York: American Public Health Association, pp. 227–247.

Kumaresan G, Annalvilli R and Sivakumar K (2007) Psychrotrophic spoilage of raw milk at different temperatures of storage. *Journal of Applied Sciences Research* **3**, 1383–1387.

Louws FJ, Fulbright DW, Stephens CT and Debruijn FJ (1994) Specific -genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas pathovars* and strains generated with repetitive sequences and PCR. *Applied and Environmental Microbiology* **60**, 2286–2295.

Mahieu H (1991) Modificaciones de la leche después de su recogida. In Luquet FM (ed.), *Leche y productos lácteos. la leche de la mama a la lechería*. Zaragoza: Acribia, pp. 22–181.

Scarpellini M, Franzetti L and Galli A (2004) Development of PCR assay to identify *Pseudomonas fluorescens* and its biotype. *FEMS Microbiology Letters* **236**, 257–260.

Spilker T, Coenye T, Vandame P and Lipuma JJ (2004) PCR-Based assay for differentiation of *Pseudomonas aeruginosa* from other *Pseudomonas* species recovered from cystic fibrosis patients. *Journal of Clinical Microbiology* **42**, 2074–2079.

Vidal AMC, Netto AS, Vaz ACN, Capodifóglio E, Gonçalves ACS, Rossi GAM, Figueiredo AS and Ruiz VLA (2017) *Pseudomonas* spp.: contamination sources in bulk tanks of dairy farms. *Pesquisa Veterinaria Brasileira* **37**, 941–948.

Yamamoto S and Harayama S (1995) Amplification and direct sequencing of gyrB genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. *Applied and Environmental Microbiology* **61**, 1104–1109.

https://doi.org/10.1017/S0022029922000048 Published online by Cambridge University Press