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Lipolytic and proteolytic activity of *Pseudomonas* spp. isolated during milking and storage of refrigerated raw milk

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ABSTRACT

The aim of this study was to verify the presence of lipolytic and proteolytic *Pseudomonas* spp. during milking and storage of refrigerated raw milk. We also intended to compare samples collected during rainy and dry seasons, from farms with manual and mechanical milking systems. For this, samples of milkers' hands, cows' teats, water, expansion tanks, equipment, and utensils used during milking were analyzed regarding *Pseudomonas* spp. count. Positive samples were tested for the production of lipolytic and proteolytic enzymes. Microorganisms of the genus *Pseudomonas* were isolated from all sampling points. A higher isolation rate of the bacterium was found in the rainy season except for 6 sampling points, with all of these associated with mechanical milking systems. *Pseudomonas* spp. exhibiting lipolytic activity were found to be predominant during the dry season, since no activity was detected during the rainy season in 26 of the 29 sampling sites. The highest number of lipolytic *Pseudomonas* isolates was obtained from water. Presence of lipase-producing *Pseudomonas* spp. was verified in 7 and 36% of the samples collected from farms with manual and mechanical milking, respectively. When analyzing raw milk collected from expansion tanks immediately (0 h) and 24 h after milking, we observed that for dairy properties with manual milking process, 10% of the *Pseudomonas* isolates were positive for lipolytic activity. The percentage increased to 12% 48 h after milking. Mean averages were 32, 33, and 39% immediately after, 24 and 48 h after milking, respectively, for farms with mechanical milking. All sampling points showed the presence of proteolytic strains of *Pseudomonas*. The highest proteolytic activity was found during the rainy season, except for the samples collected from milkers' hands before milking, buckets, and teat cup inner surfaces after milking and from the water in dairy farms with mechanical milking system. Of these samples, 72, 56, and 50%, respectively, were positive for proteolysis during the dry season. For the water samples, a statistical difference was observed between mechanical (50%) and manual (7%) milking systems in the percentage of proteolytic activity. No production of proteolytic enzyme was detected in the samples from milkers' hands taken after milking and no statistically significant difference was found among manual (19.91%) and mechanical (47.85%) milking. During the rainy months, no proteolysis was detected in the samples taken from cows' teats after the predipping. It is evident, therefore, that preventive measures capable of minimizing the contamination with *Pseudomonas* spp. during milking and storage of refrigerated raw milk are needed, regardless of season.
Key words: milk, milking system, psychrotrophic, storage

INTRODUCTION

According to the USDA, in 2014, Brazil was the world's sixth largest milk producer behind the European Union, United States, India, China, and Russia (USDA, 2015). However, the bacteriological quality of the milk produced in Brazil is considered unsatisfactory and is a chronic problem that is difficult to solve. Problems related to the health of the mammary gland, hygiene during the milking process, effectiveness of cleaning practices, and raw milk storage conditions (Elmoslemany et al., 2009) are closely linked to the poor microbiological quality of the milk produced in the country.

From the expansion of the dairy sector in the country arose the necessity of prolonged storage of raw milk. However, with this also came the challenge of maintaining the quality of the products, preventing their deterioration and extending shelf life (Zeni et al., 2013).

Thus, in 2011, the Normative Instruction 62 (IN 62) was regulated by the Brazilian Ministry of Agriculture,

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Livestock and Supply. This states that refrigerated raw milk obtained from dairy farms must be stored in stainless-steel tanks at 4°C within less than 3 h after milking, remaining at this temperature for up to 48 h after the first milking (Brasil, 2011).

Cooling the milk immediately after milking aims to reduce multiplication of mesophilic bacteria that cause deterioration due to acidifying activity (Fagundes et al., 2006; Zeni et al., 2013). However, prolonged refrigerated storage at 4°C favors the development of psychrotrophic microorganisms, which, if present in feedstock, may cause alterations in milk and dairy products (Fagundes et al., 2006) due to the production of heat-resistant enzymes even after pasteurization and UHT treatment (Zeni et al., 2013). Thereby, proper management and hygiene practices during milking, storage, and transportation of refrigerated raw milk play a critical role in its bacteriological quality (Guerreiro et al., 2005).

Santos et al. (2009) found average temperatures of 14.5, 5.5, 5.2, and 5.4°C after storing raw milk for 0, 24, 48, and 72 h, respectively. The authors concluded that the cooling temperature oscillates in dairy farms, which configures marginal refrigeration and provides favorable conditions for multiplication of psychrotrophic microorganisms (Santos and Laranja, 2001).

Psychrotrophic bacteria are defined as bacteria with high proliferation rates at low temperatures. Some strains of psychrotrophic microorganisms are known to have lipolytic and proteolytic ability (Cousin, 1982; Izidoro et al., 2013). Microorganisms of the genus *Pseudomonas* are classic psychrophiles, and according to Izidoro et al. (2013), have the highest metabolic activity, mostly lipolytic, in refrigerated milk (4 to 7°C).

Psychrotrophic microorganisms come from soil, water, air, dust, vegetation, and feces (Shah, 1994). Contact with the surface of the cooling tank, milking equipment, or utensils (or a combination of these) not properly cleaned and sanitized can contaminate the milk because these microorganisms easily multiply in milk residues found in containers, rubbers, joints, and any other location where accumulation of milk residues occurs (Guerreiro et al., 2005).

Even before the milking process, the teat skin of cows may be contaminated with psychrotrophic bacteria able to multiply at low temperatures and produce deteriorating enzymes, along with thermoduric microorganisms, which are resistant to pasteurization (Desmasures and Gueguen, 1997; Arcuri et al., 2008).

According to Forsythe (2002), bacteria of the genus *Pseudomonas* produce exopolysaccharides that contribute to biofilm formation and adhesion to surfaces of equipment and utensils used in the processing of milk and dairy products. Biofilms contain proteins, lipids, carbohydrates, minerals, and vitamins that form a crust under which bacteria continue to multiply, forming a pure culture or in association with other microorganisms that make them more resistant to the action of chemical and physical agents used for hygienization (Mosteller and Bishop, 1993; Parizzi, 1998; Nörnberg, 2009). Thus, when failures in hygiene procedures occur, residues adhere to equipment and surfaces become a potential source of contamination of milk (Nörnberg, 2009).

Lipolytic ability of *Pseudomonas* spp. depends on lipases secreted by these microorganisms that act mainly on the α portion of triglycerides (Castberg, 1992; Guerreiro et al., 2005), conferring a bitter taste and unpleasant aroma, characteristic of short-chain fatty acids, to the milk resulting in outright rejection of the milk by the dairy industry. The milk is then used by the cosmetics and soap industry (Nörnberg, 2009).

The proteolytic activity is carried out by the action of enzymes called heat-resistant proteases, naturally present in milk or produced by bacteria, particularly the psychrotrophic group, acting on κ-casein. On the other hand, whey proteins are more resistant to the action of these enzymes (Nörnberg et al., 2009). The κ-casein hydrolysis causes destabilization of milk casein micelles and consequently coagulation and gel formation, making it impossible to consume the milk (Cousin, 1982; Shah, 1994).

In view of the production systems and agro-industrial chain of milk, it is of paramount importance to analyze the critical points that can lead to contamination with psychrotrophic, establishing a relationship between refrigeration, good manufacturing and hygiene practices during milking, storage, and transportation (Zeni et al., 2013).

The aim of this study was to verify the presence as well as the lipolytic and proteolytic activity of *Pseudomonas* spp. during milking by manual and mechanical means, and storage of refrigerated raw milk in rainy and dry seasons.

MATERIALS AND METHODS

Refrigerated raw milk samples were collected from 10 dairy farms belonging to the Rural Regional of the Development Office–EDR Limeira/SP. Five properties had mechanical milking with pipe closed circuit system and 5 had manual milking systems. All farms had expansion tanks. Samples were collected during the rainy season (between December 2013 and February 2014) and during the dry season (May to August 2014) at 3 different times for each property and season. During the study period, the average rainfall in the rainy and dry seasons was 85.20 and 21.85 mm, respectively (USP, 2015).

Samples of water used to clean the milking machines, equipment, and utensils, and the expansion tanks were collected. A pool of swabs was sampled from different sites during milking process: milkers' hands before and after milking, surface of the cows' teats before and after pre-dipping, inner surface of teat cups (mechanical milking) or bucket (manual milking) before and after milking, and inner surface of the expansion tank before milking.

The analyses were performed at the Animal Health and Food Security Multi-User Laboratory, School of Animal Science and Food Engineering, University of Sao Paulo.

Pseudomonas agar base (CM 559-Oxoid; Oxoid, Basingstoke, Hampshire, UK) with addition of supplement (CFC-SR103-Oxoid) was used for enumeration of *Pseudomonas* spp. (Cousin and Bramley, 1981). Serial decimal dilutions (up to 10^{-3}) in 0.1% peptone water were prepared and 0.1-mL samples of appropriate dilutions were plated and then spread with a Drigalski handle. The plates were incubated at 28°C for 48 h. Colonies were counted with a colony counter, and the total was multiplied by the plate dilution factor.

After counting, 3 to 5 colonies characteristic of *Pseudomonas* spp. were seeded in 5 mL of brain heart infusion broth and incubated at 35°C for 18 h (APHA, 1992). Then, 0.1 mL was plated on tributyrin agar (plate count agar supplemented with 1% tributyrin) and incubated at 28°C for 5 d to test for lipolytic activity (Beerens and Luquet, 1990).

To evaluate the proteolytic activity (Beerens and Luquet, 1990) of *Pseudomonas* spp., 0.1 mL of the inoculate was plated on milk agar (plate count agar supplemented with 1% skim milk powder) and then incubated at 28°C for 24 to 48 h. In both tests performed, positivity was verified by the formation of a transparent halo around the colony.

A general linear model was used to examine the differences in *Pseudomonas* spp. proportions between different milking systems and period of year. As recommended by Banzatto and Kronka (2006), we used the scale of transformation "natural logarithm of the number of CFU + 1 (NL_CFU)" and then the ANOVA to assess the presence of *Pseudomonas* spp. Statistical analysis were carried out using the PROC MIXED procedure of the Statistical Analysis System version 9.1.3. (SAS Institute Inc., Cary, NC). The following model was adopted:

$$Y_{ijk} = \mu + T_i + E_j + TE_{ij} + P_k + e_{ijk},$$

where Y_{ijk} = number of colony-forming units in transformed scale in farm k, season j, and milking system i; μ = constant inherent to all observations; T_i = fixed effect of the ith milking system, where i = 1 (mechanical) or 2 (manual); E_j = fixed effect of the jth season, where j = 1 (dry) or 2 (rainy); P_k = random effect of the farm, supposed normally, independently identically distributed (**NIID**) $\left(0, \sigma_p^2\right)$; TE_{ij} = effect of double interaction existing between milking system i and season j; and e_{ijk} = residual random effect associated with the number of colony-forming units in transformed scale in farm k, season j, and milking system i, supposed NIID $\left(0, \sigma_p^2\right)$.

Season effect was removed when *Pseudomonas* spp. were isolated in only one season of the year (dry or rainy).

A generalized linear model (assuming binomial distribution and logistic function) was used to assess the presence or absence of *Pseudomonas* spp. with lipolytic or proteolytic ability, according to the different milking systems and seasons. Data were analyzed using the PROC GLIMMIX procedure of the Statistical Analysis System version 9.1.3. (SAS Institute Inc.). The following model was adopted:

$$Y_{ijk} = \eta(x) = \mu + T_i + E_j + TE_{ij} + P_k + e_{ijk},$$

where Y_{ijkl} = percentage of *Pseudomonas* isolates with lipolytic or proteolytic activity in farm k, season j, and milking system i; η = logistic function relating the binomial variable with the systematic component of the model; μ = constant inherent to all observations; T_i = fixed effect of the ith milking system, where i = 1 (mechanical) or 2 (manual); E_j = fixed effect of the jth season, where j = 1 (dry) or 2 (rainy); P_k = random effect of the farm, supposed NIID $\left(0, \sigma_p^2\right)$; TE_{ij} = effect of double interaction existing between milking system i and season j; e_{ijk} = residual random effect associated with farm k, season j, and milking system i, supposed NIID $\left(0, \sigma_p^2\right)$.

Season effect was removed when *Pseudomonas* spp. exhibiting lipolytic or proteolytic activity was detected in only one season of the year (dry or rainy).

RESULTS AND DISCUSSION

Isolation and Enumeration of Pseudomonas spp.

Table 1 shows the counts of *Pseudomonas* spp. during the rainy and dry seasons. In the rainy months, we found a minimum count of 1.09×10^1 cfu·mL^{-1} in the water used in the milking parlor and a maximum count of 5.40×10^4 cfu·mL^{-1} was detected on the surface of cows' teats after pre-dipping when manual milking was

Table 1. Average counts of *Pseudomonas* spp. enumerated during milking according to the interactions between milking system (manual and mechanical) and season (rainy and dry)[1]

Sampling site	Milking system	*Pseudomonas* spp. (cfu·mL^{-1})	
		Rainy season	Dry season
Water	Manual	1.92×10^{2} A,a	3.25×10^{1} A,a
	Mechanical	1.09×10^{1} A,a	2.33×10^{1} A,a
Milkers' hands before milking	Manual	8.44×10^{2} A,a	1.30×10^{1} B,a
	Mechanical	1.19×10^{2} A,a	2.61×10^{1} A,a
Milkers' hands after milking	Manual	2.48×10^{3} A,a	3.07×10^{1} B,a
	Mechanical	1.10×10^{2} A,a	1.06×10^{2} A,a
Teat surface before pre-dipping	Manual	1.08×10^{4} A,a	4.05×10^{2} B,a
	Mechanical	1.19×10^{2} A,b	4.11×10^{2} A,a
Teat surface after pre-dipping	Manual	5.40×10^{4} A,a	1.97×10^{1} B,a
	Mechanical	1.65×10^{2} A,a	1.11×10^{3} A,a
Bucket/teat cups inner surface before milking	Manual	5.86×10^{3} A,a	3.84×10^{1} B,b
	Mechanical	1.22×10^{3} A,a	1.19×10^{3} A,a
Bucket/teat cups inner surface after milking	Manual	1.28×10^{3} A,a	1.70×10^{1} B,b
	Mechanical	3.67×10^{2} A,a	1.50×10^{3} A,a
Expansion tank inner surface before milking	Manual	4.10×10^{4} A,a	3.01×10^{3} A,a
	Mechanical	9.24×10^{2} A,b	8.50×10^{3} A,a
Sieve before milking	Manual	1.79×10^{3} A	7.27×10^{2} A
Sieve after milking	Manual	5.99×10^{3} A	5.78×10^{2} A
Milk can before milking	Manual	4.12×10^{3} A	1.17×10^{3} A
Milk can after milking	Manual	6.05×10^{3} A	1.03×10^{4} A

[A,B;a,b]Values in the same row with different uppercase letters (dry season vs. rainy season) and the same column with different lowercase letters (mechanical milking vs. manual milking) were significantly different ($P < 0.05$).

[1]Samples were collected in farms belonging to the Rural Regional of the Development Office, EDR Limeira, SP, from December 2013 to August 2014.

performed. In the dry season, a minimum average count of 1.3×10^{1} cfu·mL^{-1} was observed in samples collected from milkers' hands before milking and maximum counts, found on milk cans after milking, averaged 1.03×10^{4} cfu·mL^{-1}.

Bacteria of the genus *Pseudomonas* were isolated from all sampling points, highlighting the need to intensify protective measures to minimize contamination by these microorganisms, which may cause various technological flaws in the dairy industry due to production of thermostable lipolytic and proteolytic enzymes.

Except for the samples collected from water (2.33×10^{1} cfu·mL^{-1}), teats before and after pre-dipping (4.11×10^{2} cfu·mL^{-1} and 1.11×10^{3} cfu·mL^{-1}, respectively), internal surface of teat cups and milk cans after milking (1.50×10^{3} cfu·mL^{-1} and 1.03×10^{4} cfu·mL^{-1}, respectively), and from expansion tanks inner surface before milking (8.50×10^{3} cfu·mL^{-1}), *Pseudomonas* spp. counts were higher during the rainy season ($P < 0.05$). All of these values mentioned above refer to samples collected from farms with a mechanical milking system and in the dry season. This indicates that *Pseudomonas* spp. are of environmental origin and grow in humid environments as stated by Costa et al. (1996), who reported the predilection of the *Pseudomonadaceae* family for aquatic habitats, wetlands, and areas with high rainfall. Silva et al. (2010) showed that during the rainy season, the herd health, water quality, hygiene conditions of the cattle stalls, milking equipment, and milkers' hands contributed decisively to the highest psychrotrophic count, especially *Pseudomonas* spp.

The water used in most of the properties where the samples were collected came from underground springs and did not receive any treatment before its utilization. Several authors highlight the importance of microbiological quality of water used in dairy farms, given that it comes into contact with all milking equipment and utensils and with milkers' hands before milking, favoring formation of biofilms and cross-contamination (Cousin and Bramley, 1981; Desmasures and Gueguen, 1997; Santana et al., 2001; Fagundes et al., 2006; Nörnberg, 2009).

Pseudomonas spp. levels in the water samples collected from farms with manual milking averaged 1.92×10^{2} cfu·mL^{-1} and 3.25×10^{1} cfu·mL^{-1} during the rainy and dry season, respectively, and 1.09×10^{1} cfu·mL^{-1} and 2.33×10^{1} cfu·mL^{-1} in properties with mechanical milking systems. Because no statistically significant difference was observed, we suggest that microbiological quality of water is similar between milking systems and seasons with respect to the count of *Pseudomonas* spp.

Fagundes et al. (2006) found mean counts of 1.49×10^{4} cfu·mL^{-1} in water used in farms with poor hygiene conditions. These results are much higher than those

obtained by us (maximum count of 1.92×10^2 cfu·mL^{-1} in water samples of farms with manual milking during rainy season).

Fagundes et al. (2006) also reported statistically significant differences in *Pseudomonas* spp. levels on the hands of milkers between farms with proper (4.36×10^3 cfu·mL^{-1}) and poor (6.26×10^3 cfu·mL^{-1}) hygiene measures. However, in both cases, contamination with *Pseudomonas* spp. was considered high, highlighting the importance of efficient hygiene practices, regardless of the type of milk produced. In this study, we verified a higher contamination in milkers' hand samples collected before and after milking in farms with manual milking and in the rainy season, which may be explained by the fact that *Pseudomonas* spp. is an environmental contaminant (Shah, 1994). During the rainy season, the challenges with hygiene generally increase, especially in properties with manual milking systems in which the milker is in constant contact with the straps used for containment of animals and with the teat surface, representing a potential source of contamination for the milk.

No statistically significant difference in *Pseudomonas* spp. counts was noted between manual and mechanical milking during the dry season for the samples collected from milkers' hands before the milking process. However, a greater number of microorganisms was isolated in farms with mechanical milking systems during the dry season, probably due to contamination of the milking parlor water supply during the cleaning of the milking machines and cows' teats.

Upon comparing the dry and rainy season, the latter showed a statistically significantly higher level ($P < 0.05$) of *Pseudomonas* spp. on the surface of the teats before (1.08×10^4 cfu·mL^{-1}) and after (5.04×10^4 cfu·mL^{-1}) pre-dipping in farms with manual milking. Low-humidity environments pose a major challenge for growth and development of *Pseudomonas* spp. For the samples collected from the teats before pre-dipping in the rainy season, we also observed a statistical significant difference ($P < 0.05$) between milking systems (manual: 1.08×10^4 cfu·mL^{-1} vs. mechanical: 1.19×10^2 cfu·mL^{-1}). The generally poor hygiene during milking in farms with manual system, where only unchlorinated water was used to clean the teats, may justify these findings. In only 2 properties with mechanical milking, iodine solution was used to clean the teats, whereas in the others pre-dipping was done with unchlorinated water. This may explain the increase in the count of *Pseudomonas* spp. on cows' teats after pre-dipping compared with samples taken before pre-dipping in farms with mechanical milking during the dry season.

In this study, pre-dipping was not effective for the control of bacteria of the genus *Pseudomonas* in the farms surveyed. However, according to Oliveira et al. (2010), pre-dipping helps to prevent the contamination of milking equipment by environmental microorganisms. Fernandes et al. (2009) noted the presence of *Pseudomonas* spp. in the pre-dipping solution, thus demonstrating the transmission of these microorganisms among cows. The authors affirmed that *Pseudomonas* spp. contamination increased after the use of pre-dipping in the dairy farms assessed, showing that commercial iodine solution (4 ppm) is not efficient in eliminating *Pseudomonas* spp.

Although not statistically significant, in the rainy season, the initial count of *Pseudomonas* spp. was higher in samples from the bucket surface compared with the teat cups both before and after milking. A reversed statistically significant relationship was observed during dry months ($P < 0.05$, Table 1). According to Guerreiro et al. (2005), presence of residual milk in the milking unit is favorable to growth and development of these bacteria. Fagundes et al. (2006) reported an increased presence of *Pseudomonas* spp. in dairy farms with poor hygiene standards. This may be related to inadequate practices in cleaning and sanitizing of surfaces/equipment. Santana et al. (2001) found that using hot water (100°C) in the cleaning process of teat cups is beneficial in reducing the initial contamination with mesophilic and psychrotrophic microorganisms.

Improper cleaning practices after milk collection by bulk tanker could also explain the contamination of the inner surface of expansion tanks. When not removed in the cleaning process, microorganisms attach to the surface of milking equipment and utensils favoring the formation of biofilms, which are resistant to sanitization and may serve as a continuous contamination source (Figueiredo et al., 2009).

No statistically significant difference in the count of *Pseudomonas* spp. was observed for samples collected from sieves and milk cans before and after milking. Mean averages of *Pseudomonas* spp. for the sieve surface before milking were 1.79×10^3 and 7.27×10^2 cfu·mL^{-1} in the rainy and dry season, respectively. As for samples collected after milking, *Pseudomonas* spp. counts averaged 5.99×10^3 and 5.78×10^2 cfu·mL^{-1}. For samples taken from milk cans before and after milking, average *Pseudomonas* spp. counts were of 12×10^3 and 6.05×10^3 cfu·mL^{-1}, respectively, in the rainy season, and 1.17×10^3 and 1.03×10^4 cfu·mL^{-1} during the dry season. In all cases, values were considered elevated, thus contributing to further contamination of milk. Once again, the need for preventive measures capable of controlling *Pseudomonas* spp. load on milking machines and utensils was highlighted.

The milk from the expansion tank was sampled at 0, 24, and 48 h after the mechanical milking, and at 0,

Table 2. Average counts of *Pseudomonas* spp. enumerated during storage of refrigerated raw milk according to the interactions between milking system (manual and mechanical) and season (rainy and dry)[1]

Storage period	Milking system	*Pseudomonas* spp. (cfu·mL^{-1})	
		Rainy season	Dry season
Milk from expansion tanks immediately after milking (0 h)	Manual	$1.46 \times 10^{4\ A,a}$	$5.56 \times 10^{3\ A,a}$
	Mechanical	$6.25 \times 10^{3\ A,a}$	$4.25 \times 10^{3\ A,a}$
Milk stored for 24 h in expansion tanks	Manual	$1.69 \times 10^{4\ A,a}$	$4.15 \times 10^{3\ A,a}$
	Mechanical	$1.72 \times 10^{4\ A,a}$	$6.21 \times 10^{3\ A,a}$
Milk stored for 48 h in expansion tanks	Manual	$4.67 \times 10^{4\ A,a}$	$9.16 \times 10^{3\ A,a}$
	Mechanical	$3.57 \times 10^{4\ A,a}$	$3.48 \times 10^{3\ A,a}$
Milk stored for 72 h in expansion tanks	Manual	$8.15 \times 10^{4\ A}$	$1.93 \times 10^{4\ A}$
Milk stored for 96 h in expansion tanks	Manual	$1.89 \times 10^{6\ A}$	$5.78 \times 10^{4\ B}$

[A,B;a]Values in the same row with different uppercase letters and the same column with different lowercase letters were significantly different ($P < 0.05$).

[1]Samples were collected in farms belonging to the Rural Regional of the Development Office, EDR Limeira, SP, from December 2013 to August 2014.

24, 48, 72, and 96 h after manual milking. At all times, milk samples from expansion tanks were held at 4°C. Levels of *Pseudomonas* spp. enumerated during storage of refrigerated raw milk in the rainy and dry season are summarized in Table 2. During the rainy months, the minimum isolation level of *Pseudomonas* spp. (6.25×10^3 cfu·mL^{-1}) was found in milk stored immediately after milking (0 h) and the maximum averages (1.89×10^6 cfu·mL^{-1}), after storing for 96 h. In the dry season, the minimum counts (3.48×10^3 cfu·mL^{-1}) were observed in the refrigerated raw milk after 48 h, and maximum (5.78×10^4 cfu·mL^{-1}), after 96 h.

For samples collected in dairy farms with manual milking system, initial levels of *Pseudomonas* spp. at 0 h were 1.46×10^4 and 5.56×10^3 cfu·mL^{-1} in the rainy and dry season, respectively. In farms with mechanical milking, the initial level was 6.25×10^3 and 4.25×10^3 cfu·mL^{-1} at the same period. High initial counts are a strong indicator of lack of good hygiene practices. According to Cousin and Bramley (1981), the presence of feces, mud, and bedding material on the surface of cows' teats not cleaned properly is a major risk factor for the contamination of milk.

We noted an increase in the counts of *Pseudomonas* spp. during storage of milk. The same pattern was reported by Muir (1996), Ryser (1999), Santos and Laranja (2001), and Fagundes et al. (2006). However, no statistically significant difference was found between milking procedures until storage for 48 h, when the milk from dairy farms with mechanical milking was transported to milk processing plants. We found much higher counts of *Pseudomonas* spp. in milk stored for 96 h in expansion tanks during the rainy season compared with the dry season ($P < 0.05$). This shows that is crucial for the dairy companies to collect the milk within 48 h after milking, thus avoiding the high multiplication of *Pseudomonas* spp., which can cause alterations in milk composition and other characteristics.

Our results agree with the findings of Guerreiro et al. (2005) who concluded that the technology level of milking procedure does not necessarily imply milk with better microbiological quality. The researchers found higher initial counts of psychrotrophic bacteria in farms with mechanical milking systems than in properties with rudimentary manual milking. This is justified because milking machines become potential sources of milk contamination when improperly sanitized (Guerreiro et al., 2005).

Lipolytic Activity

Data presented in Tables 3 and 4 illustrate the comparison between the 2 different milking systems (manual and mechanical) in the dry season. Of all isolates tested in this study, no lipase activity was detected in 26 of the 29 sampling sites during the rainy season. In farms with manual milking, lipolytic *Pseudomonas* spp. was isolated after milking in samples of the bucket's inner surface (7.6% of total samples), milk from the expansion tank (6.6% of total samples), and milk cans (7.6% of total samples). For dairy properties with a mechanical milking system, the presence of lipolytic *Pseudomonas* spp. was not documented during the rainy months.

The results observed for the lipolytic enzymes produced by bacteria of the genus *Pseudomonas* in the dry season disagree with what is generally seen in other studies, because the bacterium belonging to the *Pseudomonadaceae* family is often associated with aquatic environments, wetlands, and high rainfall (Costa et al., 1996). Similar results were obtained by Moreira and Montanhini (2014). These authors reported lipolytic activity in 44.1% of total milk samples analyzed, whereas proteolytic activity was detected in only 11%.

Table 3. Percentage of samples collected in different sampling sites during the dry season positive for lipolytic *Pseudomonas* spp.

Sampling site	Milking system	% of samples positive for lipase positive *Pseudomonas* spp.
Water	Manual	7[b]
	Mechanical	36[a]
Milkers' hands before milking	Manual	21[a]
	Mechanical	24[a]
Milkers' hands after milking	Manual	12[a]
	Mechanical	24[a]
Teat surface before milking	Manual	10[a]
	Mechanical	16[a]
Bucket/teat cups inner surface before milking	Manual	13[a]
	Mechanical	32[a]
Bucket/teat cups inner surface after milking	Manual	10[a]
	Mechanical	17[a]
Expansion tank inner surface before milking	Manual	11[a]
	Mechanical	24[a]

[a,b]Values in the same column with different lowercase letters were significantly different ($P < 0.05$).

In this study, the highest percentage of lipolytic *Pseudomonas* spp. was observed in the water samples. Presence of lipase-producing *Pseudomonas* spp. was verified in 7 and 36% of the samples collected from farms with manual and mechanical milking, respectively. We also observed that the proportion of lipolytic *Pseudomonas* spp. in milk increased according to the storage period. For dairy properties with a manual milking process, 10% of the samples analyzed immediately (0 h) and 24 h after milking were positive for lipolytic activity. The percentage increased to 12% 48 h after milking. Mean averages were 32, 33, and 39% immediately after, and 24 and 48 h after milking, respectively, for farms with mechanical milking, thereby demonstrating that storage time is inversely proportional to milk quality.

These results could be explained by the fact that in production systems using mechanical milking, cows are generally milked at least twice a day. This practice leads to oscillation of milk temperature, favoring the growth of psychrotrophic *Pseudomonas* spp. and enzyme production by these microorganisms, as reported by Santos and Laranja (2001).

According to Arcuri et al. (2008), lipolytic *Pseudomonas* spp. percentages were higher at 7°C (90.9% of total samples), 10°C, and 21°C (both 81.81% of total samples), and *Pseudomonas fluorescens* showed lipolytic ability in 100% of the isolates tested at 4, 7, 10, and 21°C. In this work, percentages of lipolytic *Pseudomonas* spp. were lower when compared with those found by Arcuri et al. (2008), which may be due to the differences in milk storage temperatures. Besides this, not all *Pseudomonas* strains are equally capable of producing lipases with the same efficiency (Hantsis-Zacharov and Halpern, 2007).

Thus, it is evident that duration of the storage period in refrigerated tanks, especially at marginal temperatures (5 to 10°C), favors microbial multiplication and possible lipolysis action of lipases in the milk, causing its deterioration (Santos and Laranja, 2001).

Proteolytic Activity

The percentage of proteolytic activity of *Pseudomonas* spp. isolated from different sampling points during milking and storage of raw milk is shown in Table 5. For the dairy farms with a mechanical milking system, the highest proportion of proteolytic *Pseudomonas* spp. was associated with the rainy season. However, for the samples taken from the milkers' hands before milking, the inner surface of the buckets and teat cups

Table 4. Percentage of samples positive for lipolytic *Pseudomonas* spp. during storage of refrigerated raw milk in the dry season

Sampling period	Milking system	% of samples positive for lipase positive *Pseudomonas* spp.
Milk from expansion tanks immediately after milking (0 h)	Manual	10[b]
	Mechanical	32[a]
Milk stored for 24 h in expansion tanks	Manual	10[b]
	Mechanical	33[a]
Milk stored for 48 h in expansion tanks	Manual	12[a]
	Mechanical	39[a]

[a,b]Values in the same column with different lowercase letters were significantly different ($P < 0.05$).

Table 5. Percentage of samples collected in different sampling sites during milking positive for proteolytic *Pseudomonas* spp., according to milking system (manual and mechanical) and season (rainy and dry)

Sampling site	Milking system	% of samples positive for protease positive *Pseudomonas* spp.	
		Rainy season	Dry season
Water	Manual	$27^{A,a}$	$7^{A,b}$
	Mechanical	$33^{A,a}$	$50^{A,a}$
Milkers' hands before milking	Manual	$43^{A,a}$	$19^{A,a}$
	Mechanical	$51^{A,a}$	$72^{A,a}$
Milkers' hands after milking	Manual	19.91^{a}	—[1]
	Mechanical	47.85^{a}	—
Teat surface before pre-dipping	Manual	$40^{A,a}$	$6^{B,a}$
	Mechanical	$14^{A,a}$	$18^{A,a}$
Teat surface after pre-dipping	Manual	—	24.16^{a}
	Mechanical	—	34.34^{a}
Bucket/teat cups inner surface before milking	Manual	$46^{A,a}$	$20^{A,a}$
	Mechanical	$33^{A,a}$	$10^{A,a}$
Bucket/teat cups inner surface after milking	Manual	$71^{A,a}$	$6^{B,a}$
	Mechanical	$23^{A,a}$	$56^{A,a}$
Expansion tank inner surface before milking	Manual	$33^{A,a}$	$23^{A,a}$
	Mechanical	$67^{A,a}$	$29^{B,a}$

[A,B;a,b]Values in the same row with different uppercase letters and the same column with different lowercase letters were significantly different ($P < 0.05$).
[1]— = no activity.

after milking and from the water, higher percentages of *Pseudomonas* spp. exhibiting proteolytic activity were found during the dry period. Of these, 72, 56, and 50%, respectively, were considered positive.

From all sampling sites, we successfully isolated proteolytic *Pseudomonas* spp., highlighting the need for control measures against these microorganisms. A higher percentage of proteolytic *Pseudomonas* spp. was obtained from water samples collected during the dry season in farms with mechanical milking (50%) when compared with the manual system (7%), differing statistically from each other ($P < 0.05$). These results show the importance of treating the water used in dairy farms (e.g., by chlorination), because the microbiological quality of the water affects the entire milking system and consequently the quality of the milk produced.

No statistical difference was observed for proteolytic *Pseudomonas* strains between milking systems and seasons for samples collected from milkers' hands before milking. Yet, 72% of isolates collected in dairy farms with mechanical milking and during the dry season were found positive for proteolytic activity, representing a risk for milk quality. In the work of Moreira and Montanhini (2014), proteolytic activity was detected in only 6.67% of samples. In view of the results, we can state that appropriate hand and arm hygiene of workers is a critical point for the control of the spread of this bacterium among animals. This highlights the need for health education assistance programs for milk producers seeking to improve the quality of milk and dairy products (Moreira and Montanhini, 2014). In the present study, the milkers had the habit of washing hands before the start of milking in only one of the 10 farms evaluated.

No proteolytic enzyme was produced by isolates sampled from milkers' hands after milking in dry season and also no statistically significant difference between manual (19.91%) and mechanical (47.85%) milking was observed. The percentage of proteolytic *Pseudomonas* spp. isolated from the surface of cows' teats in farms with manual milking was significantly lower ($P < 0.05$) during the dry season (6%) in contrast to the rainy season (40%). This can be explained by cows' habit of lying down during the interval between milkings, which favors contamination of the skin of the teats and udder (Moreira and Montanhini, 2014).

No production of protease was observed in samples taken from teats after pre-dipping, which may be due to the antibacterial effect of the pre-dipping solution against *Pseudomonas* spp. However, the physiological mechanism of these bacteria and under which conditions the gene responsible for production of proteases is expressed remain to be elucidated.

We found relatively high percentages of proteolytic *Pseudomonas* spp. in samples from the inner surface of the buckets and teat cups collected before and after milking, and from the inner surface of the expansion tank taken before milking in the rainy period. This suggests that hygiene failures, involving incorrect temperature conditions and inadequate sanitizer concentration or delays in exchanges of the teat cups rubbers (Costa, 2006; Moreira and Montanhini, 2014), favor biofilm

Table 6. Percentage of samples collected during storage of refrigerated raw milk positive for proteolytic *Pseudomonas* spp., according to milking system (manual and mechanical) and season (rainy and dry)

Storage period	Milking system	% of samples positive for protease positive *Pseudomonas* spp.	
		Rainy season	Dry season
Milk from expansion tanks immediately after milking (0 h)	Manual	$33^{A,a}$	$27^{A,a}$
	Mechanical	$43^{A,a}$	$27^{A,a}$
Milk stored for 48 h in expansion tanks	Manual	$29^{A,a}$	$16^{A,a}$
	Mechanical	$45^{A,a}$	$17^{A,a}$

^{A,a}Values in the same row with different uppercase letters and the same column with different lowercase letters were significantly different ($P < 0.05$).

formation. According to Teh et al. (2012), bacterial attachment to stainless steel apparently increases proteolysis. When analyzing biofilms, the authors reported that *Pseudomonas fluorescens* can produce proteases at 20, 30, and 37°C (Teh et al., 2012).

Percentages of proteolytic *Pseudomonas* spp. isolated from refrigerated raw milk samples stored in expansion tanks are listed in Table 6. The highest proportions were found in the rainy season and proteolysis was detected in 45% of the samples collected 48 h after milking in farms with mechanical milking systems. Samples collected from the tank 48 h after manual milking in the dry months had the lowest percentage of proteolytic *Pseudomonas* spp., only 16%.

Although no statistically significant difference was present between milk stored in tanks for 0 or 48 h, mechanical milking posed a greater risk of contamination with proteolytic *Pseudomonas* spp. compared with manual milking.

In a prior study, Moreira and Montanhini (2014) found the highest percent of proteolytic *Pseudomonas* spp. in a farm where, although water was treated with chlorine, the first 3 milk jets were not discarded. Workers did not have the habit of discarding the first 3 milk jets in any of the farms evaluated (mechanical milking system). In the dairy properties with manual milking, the corresponding volume of milk was consumed by the calf. Nero et al. (2009) emphasize that discarding the first milk jets is an important hygienic measure because this prevents milk contamination by microorganisms present on the udder and teat surface. This is crucial because milk is stored at temperatures ideal for the development of psychrotrophic microorganisms, which can cause severe alterations in milk quality.

CONCLUSIONS

Bacteria of the genus *Pseudomonas* were isolated throughout the milking process and storage of refrigerated raw milk in dairy farms with mechanical and manual milking systems and during the rainy and dry season. We concluded that these microorganisms are of environmental origin and indicate deficient hygiene during milking and failures during storage of raw milk. We also verified the lipolytic activity (mainly in the dry season) and proteolytic activity (mainly during the rainy season) of these microorganisms.

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