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SOURCES OF PSYCHROTROPHIC CONTAMINATION AND PROTEOLYSIS INDICES IN RAW MILK

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ABSTRACT

Storage of raw milk under refrigeration makes it possible to reduce operating costs for production and prevents losses of this raw material by the acidifying activity of mesophilic bacteria. Meantime, storage for prolonged periods may result in decreased quality of dairy products, due to the growth and enzymatic activity of psychrotrophic bacteria. Thus, in this study, we evaluated the presence of psychrotrophic microorganisms, *Pseudomonas* spp. and *Pseudomonasaeruginosa* tracing sources of microbiological contamination in the milking process as well as the level of proteolysis of the milk in the expansion tank. The highest contamination ($P < 0.05$) occurred with the psychrotrophic count, with maximum values of $5.11 \log \text{CFU} \cdot \text{mL}^{-1}$ a value that has hydrolytic potential for K-casein. Some dairy farms were found to have a maximum caseinomacropetide value of $142.93 \text{ mg} \cdot \text{L}^{-1}$, representing loss of quality for the industry and a raw material that is not suitable for human consumption. The points of greatest contamination were udder surface, insufflator, milking bucket and colling system. According to principal component analysis, the values obtained for the microbiological counts were not determinants of the release of caseinomacropetide.

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INTRODUCTION

Cold storage in expansion tanks selects for the growth of *Pseudomonas* (De Jongheet *et al.*, 2011) since these bacteria are able to outgrow the other bacteria present in the raw milk microbiota becoming predominant (von Neubeck *et al.*, 2015). However, Hahneet *et al.* (2019) concluded that dominance and outgrowth under refrigeration is not specific to a single psychrotrophic species but may be strain specific and may depend on the source of contamination. *Pseudomonas aeruginosa* (*P. aeruginosa*) is a psychrotrophic microorganism that presents a major problem since it decreases the yield of dairy products (due proteolysis and lipolysis). Owing to its ability to adapt, it is a potential component of biofilm formation in hulling food industries, has high scores in the water supply, and high virulence (Fernandes *et al.*, 2009).

In view of the production systems and agro-industrial chain of milk, it is of paramount importance to analyse the critical points that can lead to contamination with psychrotrophic microorganisms, establishing a relationship between refrigeration and good manufacturing and hygiene practices during milking, storage, and transportation (Zeniet *et al.*, 2013). Similarly, to chymosin (rennet), the psychrotrophic microorganisms present in raw milk, carry out the hydrolysis of casein, which may cause an increase in the level of caseinomacropetide (Magalhães, 2008). The level of serum added to milk due to fraud is determined by caseinomacropetide analysis (CMP), which quantifies the free CMP in milk (Brasil 2006). However, most dairy industries does not yet have a mandatory fraud analysis for serum addition at the receiving platform, only a density analysis, which shows only the possible presence of water (Brazil 2018; Valente *et al.*, 2014). Thus, this study aimed to

determine the points of contamination in the milking process and raw milk by evaluating the counts of psychrotrophic microorganisms, *Pseudomonas* spp., and *Pseudomonas aeruginosa*, as well as the level of proteolysis in milk by quantifying CMP in dairy farms in Paraná, Brazil.

MATERIALS AND METHODS

The study was conducted in a cooperative west of Paraná, Brazil. Dairy farms were chosen because they represent the characteristics of production and facilities commonly found in Brazil. Ten farms performed mechanical milking and one property had manual milking. In order to trace contamination sources, first, samples were collected from 11 rural producers and two collections were carried out. Swabs were taken of hands of the milkers, the insufflators (before the start of milking), the teats (after sanitising and before milking), the buckets, tank cooler, and milk collection directly from the expansion tank. The collection methodologies and the quantities were chosen according to Brasil (2017). Twenty-two samples of water were collected in sterile bottles from the milking room, representing the water used to wash the utensils (two samples per property, 300 mL each). The samples were analysed for psychrotrophs and *Pseudomonas*. The milk samples were collected at the time of collection by the milk truck, representing the milk that would benefit the industry. All of the milk samples, $N = 22$, were collected directly from expansion tanks and analysed for the presence of *Pseudomonas aeruginosa*, *Pseudomonas* spp. and psychrotrophic microorganisms. The total numbers of animals per property were: 10, 11, 15, 11, 6, 7, 4, 6, 20, 13, and 12. Four teats per animal were sampled. For the teats and teacups a sample area of 3 cm² was used, for the brasses and buckets a 100 cm² sample area was used, and for the cooler, a 500 cm² area was used. The areas used were defined with flexible sterile polyethylene molds (Santana et al., 2001). The swabs used to collect samples were non-toxic and consisted of a cellulose sponge immersed in 0.1% saline solution (3M Hydra-Sponge®). The milk collection pots were previously autoclaved at 121°C for 15 minutes, and the milk collected (500 mL) was stored under refrigeration, using stainless material flamed with 70 % ethanol. After collection, the samples were stored in an isothermal box with ice until arrival at the laboratory and were then analysed in the case of microbiological samples, or immediately frozen in the case of samples for proteolytic analysis (-18°C).

The microbiological analysis involved plating in duplicate surface dilutions 10¹–10³ with 0.1% peptone water in 9 mL tubes. The culture medium Cetrinide Agar (Himedia, India) was used at 35°C for 48 hours for the *Pseudomonas aeruginosa* count. The culture medium *Pseudomonas* Agar Base (Himedia, India) was used at 35°C for 24 hours for the *Pseudomonas* spp. count. For the quantification of psychrotrophic microorganisms, Agar PCA (Plate Count Agar) (Himedia, India) was used at 7°C for 10 days, all following the recommendations described by AOAC (1992). In the second experiment, milk tank samples from 11 properties were sampled in two collections, for microbiological quantification (Psychrotrophs, *Pseudomonas* spp. and *Pseudomonas aeruginosa*) and the relationship with proteolysis indexes. The level of proteolysis in raw milk from expansion tanks was analysed by quantifying the level of caseinomacropptide (CMP) using high-performance liquid chromatography (Brasil, 2006). The samples were thawed at 30°C in a water bath. A

volume of 20.0 mL of the thawed sample was added dropwise to 10.0 mL of 24% trichloroacetic acid with constant stirring, left to stand for 60 minutes at room temperature (25° C) and filtered. The calculation of the linear regression curve was ($Y = AX + B$), where A is the slope and B is the intersection with the Y axis and the linear coefficient using the concentration of CMP in milligrams per litre versus height or peak area. The curve was accepted for R-values > 0.95. The concentration of CMP was converted into milligrams per litre using chromatography, the samples from the calibration curve for the equation ($Y = AX + B$), and the results of samples expressed in milligrams CMP per litre (mg·L⁻¹) (Brasil 2006). A nonparametric statistical analysis was performed using a Kruskal-Wallis test to analyse the data tracking psychrotrophics in milking stages. Proteolysis data were evaluated for a pattern of distribution using a Shapiro-Wilk test, and the homogeneity of variances was tested using a Levene test. Since the data were not in accordance with the assumptions of normality and homoscedasticity, the comparison of the log counts of bacteria was performed using the nonparametric Kruskal-Wallis test. Data from bacteria counts and the CMP information were evaluated by means of Principal Component Analysis. The data of the variables were standardised and the quality of the data was verified using the Kaiser-Meyer-Olkin method (KMO test). The evaluation of the correlation matrices variables was evaluated using a Bartlett's sphericity test and setting the number of principal components was defined using a Broken-stick test. Data were analysed using ANOVA and disparity between the means was compared using a Tukey test at a significance level of 5% probability. All analyses were performed in XLSTAT software (Addinsoft, 2016).

RESULTS

For the psychrotrophic microorganisms count (Table 1), the main contaminant of the milk was the udder surface (log 3.99 CFU·mL⁻¹) and colling system (3.90 log CFU·mL⁻¹), however, the insufflator and bucket were not statistically different (3.85 and 3.75 log CFU mL⁻¹, respectively) ($P > 0.05$). In the second collection, the udder surface also presented a median count (4.32 log CFU·mL⁻¹) that could influence the contamination of the milk. The highest values for *Pseudomonas* spp. count was obtained in the first collection from the milking bucket (4.22 log CFU·mL⁻¹). In the second collection, the major contaminant was the colling system. The *Pseudomonas aeruginosa* counts was maximum count of 6.90 log CFU·mL⁻¹ for the first collection and 5.26 log CFU·mL⁻¹ for the second collection (Table 2). High bacterial counts were also seen in the second experiment, which quantified the proteolysis indices of the tank milk (Table 3). For the evaluation of bacterial counts, CMP was performed to verify that the variables were in accordance with the conditions for application of the principal component analysis (PCA) since the KMO value was greater than 0.5 (KMO = 0.565). The relationship between the matrices of variables was investigated using a Bartlett test ($\chi^2 = 28.302$, $p < 0.0001$). Using the criteria of Broken-Stick, it was possible to identify two main components as significant in the analysis. The first two ACP factors showed a cumulative variation of 71.781% (F1 = 1.906; F2 = 1.005). The first canonical axis (F1) applied to the variables (47.7% of the variability) was governed by the variation in *Pseudomonas* counts, and denotes the relationship between *P. aeruginosa* and *Pseudomonas* spp. The principal component analysis showed that higher counts of *Pseudomonas* spp. are related to higher *P. aeruginosa* counts.

Table 1. Psychrotrophic tracking in milk production

	1 st collection				2 st collection			
	Log (CFU mL ⁻¹)				Log (CFU mL ⁻¹)			
	Maximum	Average	Minimum	*Median	Maximum	Average	Minimum	*Median
Water	5.78	3.17	1.89	3.00 ^{abcde}	4.15	2.79	1.65	2.83 ^{bcdefg}
Bucket	5.85	3.62	<1.00	3.75 ^{cdef}	6.16	4.21	2.35	4.20 ^{efgh}
Insufflator	6.00	3.71	<1.00	3.85 ^{cdef}	5.75	3.64	<1.00	4.07 ^{cdefgh}
Milker's Hands	6.00	3.10	<1.00	3.26 ^{abcdef}	6.00	3.77	<1.00	4.05 ^{defgh}
System Coller	5.25	3.95	2.25	3.90 ^{cdef}	6.37	4.10	2.00	3.91 ^{efgh}
Udder	6.50	3.62	<1.00	3.99 ^{cdef}	6.75	4.21	<1.00	4.32 ^{efgh}
Milk	6.85	5.23	3.83	5.11 ^f	6.45	4.90	2.50	5.00 ^h

*Medians followed by the same letter in the columns do not differ statistically from each other (P> 0.05) by Tukey test.

Table 2. Tracking of *Pseudomonas* spp and *Pseudomonas aeruginosa* milk production

	1 st collection				2 st collection			
	Log (CFU mL ⁻¹)				Log (CFU mL ⁻¹)			
	Maximum	Average	Minimum	*Median	Maximum	Average	Minimum	Median
<i>Pseudomonas</i> spp.								
Water	6.80	3.78	<1.00	3.89 ^{cdef}	5.00	2.12	<1.00	2.55 ^{abcdef}
Bucket	6.75	3.69	<1.00	4.22 ^{cdef}	4.55	2.34	<1.00	2.56 ^{abcdef}
Insufflator	6.80	3.87	<1.00	4.06 ^{cdef}	5.48	2.29	<1.00	2.81 ^{abcdefg}
Milker's Hands	6.96	2.29	<1.00	2.80 ^{abcd}	3.98	1.76	<1.00	2.34 ^{abcd}
System Coller	6.94	3.48	<1.00	3.19 ^{cdef}	4.29	2.40	<1.00	2.90 ^{abcdefg}
Udder	6.98	3.37	<1.00	3.00 ^{bcdef}	4.50	1.48	<1.00	<1.00 ^{abc}
Milk	6.00	3.94	<1.00	4.50 ^{cdef}	6.95	2.65	<1.00	2.96 ^{bcdefg}
<i>P. aeruginosa</i>								
Water	6.80	3.78	<1.00	3.89 ^{cdef}	5.00	2.12	<1.00	2.55 ^{abcdef}
Bucket	6.75	3.69	<1.00	4.22 ^{cdef}	4.55	2.34	<1.00	2.56 ^{abcdef}
Insufflator	6.80	3.87	<1.00	4.06 ^{cdef}	5.48	2.29	<1.00	2.81 ^{abcdefg}
Milker's Hands	6.96	2.29	<1.00	2.80 ^{abcd}	3.98	1.76	<1.00	2.34 ^{abcd}
System Coller	6.94	3.48	<1.00	3.19 ^{cdef}	4.29	2.40	<1.00	2.90 ^{abcdefg}
Udder	6.98	3.37	<1.00	3.00 ^{bcdef}	4.50	1.48	<1.00	<1.00 ^{abc}
Milk	6.00	3.94	<1.00	4.50 ^{cdef}	6.95	2.65	<1.00	2.96 ^{bcdefg}

*Medians followed by the same letter in the columns do not differ statistically from each other (P> 0.05) by Tukey test.

Table 3. Average microbiological counts for ACP analysis

Bacteria (Log CFU mL ⁻¹)	Minimum	Maximum	Average	Median*	SD
1 st collection					
<i>P. aeruginosa</i>	<1.00	4.45	1.96	2.27 ^a	1.47
<i>Pseudomonas</i> spp	<1.00	6.95	2.65	2.96 ^{ab}	1.96
Psychrotrophic	1.00	5.00	3.09	5.00 ^c	0.95
2 st collection					
<i>P. aeruginosa</i>	<1.00	5.55	2.72	0 ^a	1.70
<i>Pseudomonas</i> spp	<1.00	6.00	3.94	4.50 ^{ab}	1.73
Psychrotrophic	3.83	6.85	5.23	5.11 ^c	0.78

*Medians followed by the same letter in the columns do not differ statistically from each other (P> 0.05) by Tukey test.

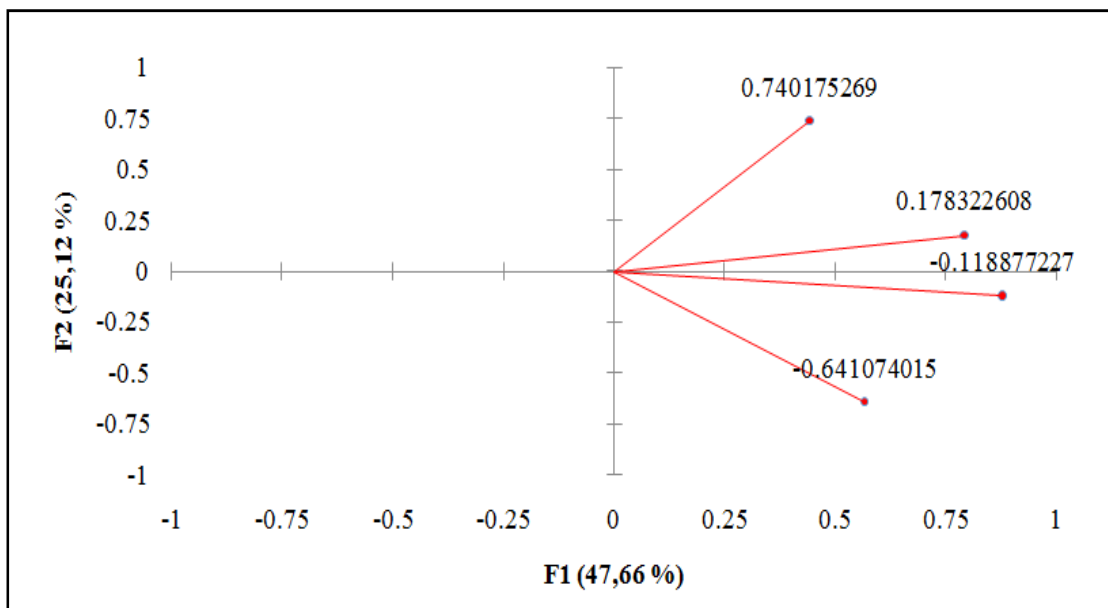


Figure 1. Ordering diagram representing the first two axes of the principal component analysis for the variables related to the count of bacteria and CMP (caseinomacropetide)

Pseudomonas spp., the higher the *P. aeruginosa* count (Table 4). The second axis was governed mainly by psychrotrophic microorganisms and CMP bacteria (25.1% of the variability). The higher the count of psychrotrophic bacteria, the smaller the value of CMP proteins (Fig 1).

Table 4. Factorial loads of the variables analyzed

	F1	F2
CMP	0.440	0.740
PAL	0.790	0.178
PSSL	0.877	-0.119
PSCL	0.566	-0.641

CMP: caseinomacropéptide; PAL: *Pseudomonas aeruginosa*; PSSL *Pseudomonas* spp.; PSCL: psychrotrophic

DISCUSSION

The use of disinfectants in pre-dipping considerably reduces microorganisms, thereby increasing the quality of milk. The farms observed in this study did not manage pre-milking hygiene correctly, and only two producers used chlorine disinfection. The other properties used only water as a hygiene measure. Only two producers used paper to dry the udders, which were the same two that used chlorine disinfection; the other producers used the same cloth or nothing to dry the udders. The water did not represent a source of psychrotrophic contamination at the points sampled, due to the low levels observed (3.00 and 2.86 log CFU·mL⁻¹). Capodifoglio et al. (2016) found that *Pseudomonas* spp. levels in water samples collected from farms were 1.90 log₁₀ and 1.37 log₁₀ in properties with manual and mechanical milking systems, respectively. In this study, high microbiological counts due to the milk cooling system are associated with the system used, since seven farms used dip tank instead of bulk tanks, currently prohibited by law (IN77). There is a global demand for dairy products with good quality and a long shelf-life. Indeed, several studies have evaluated the composition of psychrotrophic microbiota in raw milk and its deteriorating activity and have demonstrated that *Pseudomonas* is one of the most prevalent genera (Meng et al., 2018; Junior et al., 2018; Skeie et al., 2019; Yan et al., 2017) which corroborates with the data obtained in this study.

Although there are norms and legislation that regulate the sanitary, physical, and chemical quality of milk and dairy products at an international level (Codex Alimentarius), some countries, like Brazil, don't have limit for level of *Pseudomonas* and psychrotrophic microorganisms in raw milk (IN 76 and 31). According De Jonghee (2011), bacteria of the genus *Pseudomonas* were isolated from all sampling points in farms, highlighting the need to intensify protective measures to minimise contamination by these microorganisms, which may cause various technological flaws in the dairy industry due to production of thermostable lipolytic and proteolytic enzymes. The bacterium *Pseudomonas aeruginosa* possesses a high capacity for virulence, and the insufflators showed high counts (6.90 and 5.26 log CFU·mL⁻¹). *Pseudomonas aeruginosa* is an opportunistic microorganism and immune suppressed animals have a high chance of acquiring the strain, especially with the high contamination observed in this study. Oliveira et al (2009) suggest that the scores of 6 log CFU·mL⁻¹ of *Pseudomonas* result in high levels of proteolysis and a decrease in the yield of dairy products (Nörnberg, 2006), highlighting the importance of reducing microorganism levels by implementing hygiene measures in the handling of milking,

since this is the primarily source of microorganisms responsible for the production of enzymes that hydrolyse K-casein. In addition to these factors, the time until collection by the industry in these properties was relatively high; the shortest time was 48 hours and the longest was up to 96 hours. Brazil (2018) established that the collection time can be 24 hours but the maximum should be 48 hours. Santos et al. (2013) found significant microbiological differences with different storage times (24 and 96 hours), microbiological psychrotrophic values were affected by time, meaning that time is the key factor for quality, which decreases as storage time increases. The observed values of caseinomacropéptide were 0.00 mg/L to 74.283 mg L⁻¹, and extremely high values, since this milk is unsuitable for direct consumption (limit 30 mg·L⁻¹ Brazil 2006) resulting in a poor quality raw material for the industry, such values may also be related to fraud by adding serum. The level of CMP obtained correlated directly proportional with *Pseudomonas aeruginosa* e *Pseudomonas* spp. and was inversely proportional to psychrotrophic bacteria (Fig 1). It is important to emphasise that even a sample with a high CFU value will not necessarily have high levels of proteolysis of the raw material. For example, a first sample had the threshold proteolysis value of 1408.8 mg·L⁻¹ and psychrotrophic microorganism count of 5 log CFU·mL⁻¹, whereas the second collection had a proteolysis value of 33.927 mg·L⁻¹ and psychrotrophic microorganism count of 6.85 log CFU·mL⁻¹; these values indicate higher and lower levels of proteolysis, respectively. However, this study showed that the values obtained for microorganisms are not the determining factors for the release of CMP, since a higher amount of psychrotrophic bacteria had a lower level of proteolysis (Fig 1). Silva (2005) also confirmed this by isolating *Pseudomonas* spp. and evaluating proteolytic capacity and found that 60% of the isolates had proteolytic activity, thus, not all strains caused proteolysis.

Conclusion

The points of greatest contamination were udder surface, insufflator, milking bucket and colling system. The values obtained for the microbiological counts were not determinants of the release of CMP.

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