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MICROBIOLOGY

Pseudomonas spp. and *P. fluorescens*: population in refrigerated raw milk

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ABSTRACT: *Raw milk samples were collected from cooling tanks (after they cooled for 48 h) in five dairy farms and the corresponding bulk tank (bulk milk transportation, BMT) when they arrived to the industry. Routine physical chemical analyzes and quantification of psychrotrophic (**Pseudomonas** spp. and **P. fluorescens**) and aerobic mesophilic (AM) populations were performed. Only relative density and titratable acidity values for samples of milk from three farms were in agreement to the quality parameters required by law. In the BMT, only the protein content has not reached the minimum value established by law, and counting was performed for AM (>10^5 colony forming units (CFU) mL^{-1}) and psychrotrophic bacteria (2.8x10^6CFU mL^{-1}). **Pseudomonas** spp. counting corresponded to 17.9% of the psychrotrophic population, and P. fluorescens was 3.4% of **Pseudomonas spp**. count. In milk samples from dairy farms, counts were variable for AM (3.4x10^5 to 3.7 x10^7CFU mL^{-1}), psychrotrophic (4.0x10^4 to 3.1x10^6CFU mL^{-1}), **Pseudomonas** spp. (2.3x10^4 to 1.8x10^5CFU mL^{-1}), and **P. fluorescens** (62 to 8.4x10^3CFU mL^{-1}). For the populations studied, no statistical difference (P>0.05) was observed between counts reported in milk samples collected in dairy farms (cooling tanks) and BMT. Therefore, the genera **Pseudomonas** spp. and **P. fluorescens** were not the most frequent psychrotrophic bacteria in this studied milk transportation line.*
Key words: *psychrotrophic, quality, enzymes.*

Pseudomonas spp. e *P. fluorescens*: população em leite cru refrigerado

RESUMO: *Amostras de leite foram coletadas de tanques de refrigeração (após 48h de refrigeração) em cinco fazendas de produção e do respectivo caminhão tanque (leite de conjunto, LC) ao chegar à indústria beneficiadora. Análises físico químicas de rotina e quantificação das populações de bactérias psicrotróficas (**Pseudomonas** spp. e **P. fluorescens**) e aeróbios mesófilos (AM) foram realizadas. Apenas os valores de densidade relativa e acidez titulável de amostras de leite de três fazendas estavam de acordo com a legislação. No LC, apenas o teor de proteína não atingiu o valor mínimo estabelecido por lei, e a contagem foi realizada para AM (>10^5 unidades formadoras de colônias (UFC) mL^{-1}) e psicrotróficos (2,8x10^6UFC mL^{-1}). A contagem de **Pseudomonas** spp. correspondeu a 17,9% da população de psicrotróficos, e 3,4% da contagem de **Pseudomonas** spp. eram da espécie **P. fluorescens**. Nas amostras de leite das fazendas de produtoras, as contagens foram variáveis para AM (3,4x10^5a 3,7x10^7UFC mL^{-1}), psicrotróficos (4,0x10^4 a 3,1x10^6UFC mL^{-1}), **Pseudomonas** spp. (2,3x10^4 a 1,8x10^5UFC mL^{-1}) e **P. fluorescens** (62 a 8,4x10^3UFC mL^{-1}). Para as populações estudadas, nenhuma diferença estatística (P>0,05) foi observada entre as contagens encontradas nas amostras de leite coletadas nas fazendas (tanques de resfriamento) e do LC. Portanto, o gênero **Pseudomonas** spp. e a espécie **P. fluorescens** não foram os psicrotróficos mais frequentes nesta linha de transporte de leite estudada.*
Palavras-chave: *psicrotrófico, qualidade, enzima.*

INTRODUCTION

Guidelines for cooling milk in dairy farms and its transportation were established in Brazil in 2002 (BRASIL, 2002) as a response to international regulations and market demands. Since then, changes in milk chain production profile and qualitative advances in the regulation of total bacterial (TBC) and somatic cell (SCC) counts in raw milk have occurred (BRASIL, 2002), and the guidelines were updated in 2011 (BRASIL, 2011). Initially, the values for TBC and SCC should not exceed 600,000 colony forming units (CFU) mL^{-1} and 600,000 somatic cells (SC) mL^{-1}, respectively. Later (July 1, 2014), these

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parameters were changed to 300,000CFU mL^{-1} (TBC) and 500,000 cells mL^{-1} (SC) (BRASIL, 2011).

Both Ministry of Agriculture, Livestock, and Food Supply (MAPA) regulations determine that raw milk temperature at dairy farms must be 4°C or bellow. The temperatures must not exceed 7°C at dairy farm, when chilling is obtained by immersion of milk buckets or community bulk tanks, and this temperature must be reached within 3h after milking of the dairy herd is complete. Milk temperature must be at or below 10°C when the container truck arrives at the dairy plant, and its maximum time at the farm must not exceed 48 h (BRASIL, 2002; BRASIL, 2011). However, storage of raw refrigerated milk with high TBC for periods longer than 48 h at dairy farm has negatively affected dairy products mainly due to an increase in the psychrotrophic bacterial population (SANTOS et al., 2009). Psychrotrophic bacteria are mostly Gram negative bacteria, which produced heat-resistant proteolytic and lipolytic enzymes (BRAMLEY A& MCKINNON, 1996; MUIR, 1996; ANDRADE et al, 1998). Poor milking practices (VALLIN et al., 2009; YAMAZI et al., 2010; SILVA et al., 2011) and overall environmental contamination (YAMAZI et al., 2010; SILVA et al., 2011) heavily contributed to the increase in psychrotrophic species population in raw milk.

Pseudomonas spp. represents the most frequent deteriorating psychrotrophic microflora in refrigerated raw milk, and ***Pseudomonas fluorescens*** is the most frequently reported species (JAYARAO & WANG, 1999; FAGUNDES et al., 2006; ARCURI et al., 2008; SANTOS et al., 2009). Due to the changes in the entire milk production in Brazil, verifying the impact of raw milk cooling at dairy farms on ***Pseudomonas*** spp. and ***P. fluorescens*** behavior is necessary. The aim of this study was to quantitatively evaluate the presence of psychrotrophic ***Pseudomonas*** spp. and ***P. fluorescens*** in refrigerated raw milk from different dairy farms, as well as the effect of bulk milk transportation on these populations.

MATERIALS AND METHODS

Samples

Milk samples were collected from Jun 2013 to Feb 2014. Two milk samples were taken from five (A, B, C, D, and E) dairy farms in Londrina (Paraná State, Brazil), in a total of ten milk samples from individual dairy farm cooling tanks. Bulk milk transporter took only milk from the farms included in the study directly to the dairy plant, where milk samples were collected before delivery. Samples were kept in styrofoam box with reusable ice packs and brought to the Microbiology Laboratory for immediate analysis. Route length between farms and dairy plant was about 70km. At the beginning of the study, each dairy producer was asked to complete a questionnaire on good farm and milking practices.

A cooled and thoroughly homogenized milk sample (600mL) was taken from each dairy farm with a sterilized recipient. A total of 1,200mL of thoroughly homogenized milk sample was also taken directly from the bulk tank (six distinct 200-mL fractions) when the truck arrived at the plant. Milk temperature was recorded as indicated in the equipment.

Physical and chemical analysis

Protein content (%), fat content (%), non-fat solids (NFS) (%), titratable acidity (ºDornic), density (g mL^{-1}), and cryoscopy index (CI) (ºH) were determined according to the AOAC (1997). Analyses were performed in triplicate.

Microbiological analysis

Petrifilm™ AC (3M Brazil Ltda) was used as recommended by the manufacturer, but with modification (HOUGHTBY et al., 1992) to count aerobic mesophilic microorganisms. Psychrotrophic bacteria were counted by surface-plating in Plate Count Agar (Himedia, Mumbai, India) at 21°C for 25h (OLIVEIRA; PARMELLE, 1976). For ***Pseudomonas*** spp. counting, CFC-supplemented Pseudomonas Agar Base (Himedia, Mumbai, India) was used (FAGUNDES et al., 2006) at 30°C for 48h.

Tripticase in soy broth (TSB; Himedia, Mumbai, India) was used for ***P. fluorescens*** counting: each milk sample (2.5mL) was pre-incubated (21°C; 18h) in media (5mL). Then decimal dilutions were performed with the final broth, which was plated on the surface of Pseudomonas Cetrimide Agar medium (Oxoid, England) (SANTOS et al., 2009). After incubation (21°C; 48h), only the greenish-yellow colonies were counted under ultraviolet light (380nm) (KING, 1954). All microbiological results were expressed as CFU mL^{-1} of milk. All analyses were performed in duplicate.

Statistical analysis

Physical-chemical and microbiological data were evaluated using analysis of variance (ANOVA) and Tukey's test ($P<0.05$ and $P<0.10$) and the Statistica (STATSOFT, 2008) software. Results were presented as mean ± standard deviation (SD), and milk samples from farm cooling tanks and bulk milk transportation throughout the study are compared.

RESULTS AND DISCUSSION

A bulk cooling tank was used in every milk farm studied. Milking parlors had a roof and cemented floor. Farmers did not know the physical-chemical and microbiological characteristics of water used in the dairy parlors. Morning milking yields were invariably frozen in tanks of dairy farms B and C, as the equipment were overestimated to accommodate their productions (Table 1).

Among the physical-chemical data evaluated in samples from the study farms throughout the sampling period, only milk titratable acidity (14-18°D) and density (1.028-1.034g mL^{-1}; 15ºC,) met the minimum quality parameters as established by the Brazilian law on the subject (BRASIL, 2011) (Table 2). Some problems were reported at the milk dairy farm C where water addition was suspected, as indicated by the freezing point value (-0.509ºH) and decreased the NFS (8.2%) and fat contents (2.8%) (Table 2). Presence of water in the cooling tank can be responsible for the changes observed in the milk freezing point.

Minimum values were established (BRASIL, 2011) for crude protein (2.9%), fat (3%), NFS (8.4%), density (1.028-1.034g mL^{-1}), and freezing point index (from -0.530 to -0.550°H). Water addition to milk changes its freezing point, making it approach to that of water (0°C) due to dilution of milk components, especially lactose and chlorides (WALSTRA et al., 2006). Physical-chemical values for the BMT samples were within the parameters required by the Brazilian law, but the protein content was below the minimum required (2.9%). A decreased protein content was also observed among milk samples from the other dairy farms (Table 2). Although cow's nutrition was not evaluated, the protein index could be related to both the low quality of pastures and decreased dry matter in animals feeding (MARTINS et al., 2006).

Average mesophilic bacteria count were in the range $3.4x10^5$ to $3.7x10^6$CFU mL^{-1}, with no significant difference (P>0.05) between the individual populations reported in milk samples from dairy farm cooling milk tanks and the corresponding BMT (Table 3). Within the study period, only dairy farms that followed the good practices, had TBC meeting the microbiological parameters established by the federal regulation ($6.0x10^5$CFU mL^{-1}), (Table 1). However, TBC regulation was redefined on July 1, 2014 ($3.0x10^5$CFU mL^{-1}; BRASIL, 2011), and thus milk dairy farm E became close to the TBC cut-off limit, given the average count of mesophilic bacteria throughout the sampling period of this study (Table 3). Table 3 also shows that the BMT mesophilic count ($6x10^5$CFU mL^{-1}) was higher than the minimum value established by the Brazilian milk quality regulation during the studied period, as well as the current milk quality parameters (BRASIL, 2011). In general, milk produced in Brazil has low quality, as it contains high bacteria and somatic cell counts due to both poor hygiene practices during milking and inadequate sanitation (MARTINS et al., 2008; CATANIO et al., 2012; RIBEIRO JUNIOR et al., 2015).

Psychrotrophic average count in milk from study dairy farms ranged from $4.0x10^5$ to $3.1x10^7$CFU mL^{-1} (Table 3). Milk dairy farm E (Table 1) showed the lowest psychrotrophic count, which was different (P<0.05) from that of BMT. Surely, these results are due

Table 1 - Production characteristics and main milking practices in five milk farms (A, B, C, D, and E) in the Londrina region, Paraná State, Brazil, from June 2013 to February 2014.

Characteristics	Milk farms				
	A	B	C	D	E
Production (L day^{-1})	208	450	550	450	2000
Water sources	Water mine	Water mine	Artesian well	Artesian well	Water mine
Chlorinated water	N*	N	N	N	Y*
Milking System	Mechanical CS**	Mechanical CS**	Mechanical CS**	Mechanical CS**	Mechanical S**
Water leftover in the cooling tank	N	Y	Y	N	N
Use of strip cup	N***	Yes	N***	Y	Y
Use of CMT****	N***	N	N***	Y	Y
Use of predip	Y	Y	N	N	Y
Teats dried with paper towels	Y	Y	Y	Y	Y
Use of postdip	N	N	N	Y	Y

*Y=Yes;N=No; *CS= Closed system; ***test is performed only when a problem is noticed; ****CMT = California mastitis test.

Table 2 - Physical and chemical measurements (mean values)* of raw milk refrigerated samples collected from individual cooling tanks at five milk farms (A, B, C, D, and E) and the corresponding bulk milk transportation (BMT) trucks before delivery to a dairy industry in the Londrina region, Paraná State, Brazil, from June 2013 to February 2014.

Milk farms	Protein (%)	Fat (%)	NFS[C] (%)	Acidity (°D)	Density*** (g mL^{-1})	Cryoscopy index ºH)
A	2.7	4.1^{a}	8.6^{a}	14.7^{b}	1.030^{A,B}	- 0.537^{b}
B	2.8	3.5^{b}	8.5$^{a,.b}$	16.5^{a}	1.030^{A,B}	- 0.546^{a}
C	2.6	2.8^{c}	8.2^{c}	16.28^{a}	1.029^{B}	- 0.509^{c}
D	2.8	3.3^{b}	8.3^{b,c}	15.7^{a,b}	1.029$^{A,.B}$	- 0.538^{b}
E	2.9	3.5^{b}	8.5^{a,}	16.0^{a,b}	1.030^{A}	- 0.537^{b}
BMT****	2.8	3.2^{b}	8.5$^{b,.c}$	16.3^{a}	1.030^{A,B}	- 0.538^{b}

*Different lower-case letters in the same column indicates significant differences by the Tukey test (P<0.05). Different upper-case letters in the same column indicate significant differences by the Tukey test (P<0.10). **NFS = non-fat solids; ***Density was determined at 15ºC; ****BMT= bulk milk transportation. Minimum values: crude protein (2.9%), fat (3%), NFS (8.4%), density (1.028-1.034g mL^{-1}), cryoscopy index (from -0.530 to -0.550°H).

to the good practices adopted in that farm (Table 1). Some studies report off-flavor in dairy products when raw milk TBC is over 10^{6}CFU mL^{-1} (MUIR, 1996; SANTOS et al., 2009). High percentage of psychrotrophic bacteria indicates poor hygiene during milking and/or faulty procedures during storage and transportation of cooled milk. In proper sanitary conditions, psychrotrophic count is lower than 10% of the total microbiota in raw milk. However, the psychrotrophic microorganisms rate can exceed 75% when handling is faulty (SUHREN, 1989).

Pseudomonas spp. average count in milk from dairy farms ranged from 2.3x10^{5} to 1.7x10^{6}CFU mL^{-1} (Table 3). ***P. fluorescens*** average count ranged from 6.2x10^{2} to 8.4x10^{4}CFU mL^{-1}. Similar findings were also reported in milk from dairy farms where good milking practices have not been implemented (1.6x10^{5}CFU mL^{-1} of ***Pseudomonas*** spp.), and poor water quality is pointed as the key factor for the high bacterial count (FAGUNDES et al., 2006). ***Pseudomonas*** spp. average count in milk dairy farm E was lower (P<0.05) than in the BMT reported in the five dairy farms studied. According to FAGUNDES et al. (2006), the average count of ***Pseudomonas*** spp. in milk from dairy farms with poor hygiene handling is higher than in those where milking practices are appropriate.

Pseudomonas spp. are the Gram negative species most commonly reported in cooled raw milk (JAYARAO & WANG, 1999; FAGUNDES et al., 2006; ARCURI et al., 2008). However, this was not observed in this study (Table 4). ***Pseudomonas***

Table 3 - Average bacterial count (CFU mL^{-1}) in raw milk samples from individual refrigerated cooling tanks of five milk farms (A, B, C, D, and E) and the bulk milk transportation (BMT) before delivery to a dairy industry in the region of Londrina, Paraná State, Brazil, from June 2013 to February 2014.

A	B	C	D	E	Mean counts†	BMT*
----------Temperature (ºC)----------						
3.1	4.0	3.3	3.4	3.3	3.4	6.6
----------Mesophilic bacteria (CFU mL^{-1})----------						
1.6x10$^{6\ a,b}$	3.7 x10$^{6\ a}$	3.5 x10$^{6\ a,b}$	8.4 x10$^{5\ b}$	3.4 x10$^{5\ b}$	2.0 x10$^{6\ A}$	1.4 x10$^{6\ a,b,A}$
----------Psychrotrophic bacteria (CFU mL^{-1}) **----------						
1.68 x10$^{6\ a}$	9.5 x10$^{6\ a}$	3.1 x10$^{7\ a}$	1.5 x10$^{6\ a,b}$	4.0 x10$^{5\ b}$	8.9 x10$^{6\ A}$	2.8 x10$^{6\ a,A}$
----------***Pseudomonas*** spp. (CFU mL^{-1}) **----------						
6.6 x10$^{5\ a}$	1.8 x10$^{6\ a}$	1.7 x10$^{6\ a,b}$	2.3 x10$^{5\ a,b}$	2.5 x10$^{5\ b}$	9.3 x10$^{5\ A}$	5.1 x10$^{5\ a,A}$
----------***P. fluorescens*** (CFU mL^{-1}) **----------						
6.0 x10$^{3\ a}$	8.4 x10$^{4\ a}$	6.2 x10$^{2\ a}$	3.3 x10$^{3\ a}$	1.4 x10$^{3\ a}$	1.9 x10$^{4\ A}$	1.7 x10$^{4\ a,A}$

*BMT= bulk milk transportation **Different lower-case letters in the same row indicate significant difference by the Tukey test (P<0.05). Identical upper-case letters in the same line indicate non-significant difference (P>0.05).† Mean counts in milk from dairy farms.

Table 4 - Percent ratios of microorganisms reported in raw milk samples collected in cooling tanks of five milk farms (A, B, C, D, and E) and bulk milk transportation (BMT) to a dairy industry in the region of Londrina, Paraná State, Brazil, from June 2013 to February 2014.

Milk farms	***Pseudomonas*** spp. / ***Psychrotrophic*** (%)	***P. fluorescens*** / ***Pseudomonas*** spp. (%)
A	41.84	0.91
B	18.74	4.73
C	5.40	0.04
D	15.20	1.42
E	62.47	0.56
BMT*	17.93	3.42

*BMT= bulk milk transportation.

spp. count was 17.9% of the entire psychrotrophic population, whereas ***P. fluorescens*** was 3.4% of the ***Pseudomonas*** spp. population (Table 4) in the BMT milk delivered to the dairy industry. Another study conducted in Brazil (ARCURI et al., 2008) reported that in cooled raw milk ***Pseudomonas*** spp. count was 43% of the total psychrotrophic Gram negative strains, and ***P. fluorescens*** was the most common specie (37.6%), which was followed by ***P. putida***. Conversely, ***P. fluorescens*** was the predominant species (29.9%) in cooled raw milk in the USA (JAYARAO & WANG, 1999). The time elapsed between sampling of cooled raw milk at the farms and its arrival to the dairy plant was 6 h. However, the authors observed that milk remained in the bulk tank for up to 10h, was stored in the cooling tanks for more than 48h on many occasions. It is well established that temperature and storage period are crucial, especially regarding psychotropic microorganisms, whose population increase over time even under refrigeration (NÖRNBERG et al., 2009; SANTOS et al., 2009). However, no difference (P>0.05) was observed between the milk average study populations in dairy farms and BMT arrival to the industry. Therefore, short-distance routes such the one studied here may not be determinant for overall milk bacterial count. However, initial milk microbiological status at dairy farms play an important role in the average bacterial count in milk delivered to dairy industry.

CONCLUSION

The genus ***Pseudomonas*** spp. and the species ***P. fluorescens*** were not the most frequent psychrotrophic bacteria in cooled milk. Bulk milk transportation did not affect the population of mesophilic and psychrotrophic bacteria such as ***Pseudomonas*** spp. and ***Pseudomonas fluorescens*** in raw milk received by the dairy industry.

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