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Pseudomonas spp.: contamination sources in bulk tanks of dairy farms[1]

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ABSTRACT.- Vidal A.M.C., Saran Netto A., Vaz A.C.N., Capodifóglio E., Gonçalves A. C.S., Rossi G.A.M., Figueiredo A.S. & Ruiz V.L.A. 2017. ***Pseudomonas* spp.: contamination sources in bulk tanks of dairy farms.** *Pesquisa Veterinária Brasileira 37(9):941-948*. Departamento de Medicina Veterinária, Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Avenida Duque de Caxias Norte 225, Pirassununga, SP 13635-900, Brazil. E-mail: letticie@usp.br

This study focused on isolating *Pseudomonas* spp. during milking process in ten dairy farms with manual and mechanical milking systems during dry and rainy seasons, and evaluating DNA homology and patterns of distribution between isolates, in order to identify main sources of milk contamination by *Pseudomonas* spp. A total of 167 isolates of *Pseudomonas* spp. were obtained from water, milkers' hands, cows' teats, teat cups, cooling tanks and raw milk. Bacteria of *Pseudomonas* spp. genus were isolated from 85 and 82 sampling points in dairy farms with manual and mechanical milking system, respectively. A significant difference (p=0.02) on *Pseudomonas* spp. isolation was observed among samples of surface of cows' teats before and after pre-dipping, but no significant difference (p>0.05) was observed among milking systems or seasons. The possibility of the same *Pseudomonas* spp. patterns are distributed in different farms and seasons using Amplified Fragment Length Polymorphism (AFLP) technique was demonstrated. Milkers' hands, surface of cows' teats, teat cups and cooling tanks were associated with raw milk contamination with *Pseudomonas* spp. on farms with manual and mechanical milking system, showing that regardless of the type of milking system and season, proper hygiene procedures of equipment, utensils and workers' hands are essential to avoid contamination of the milk and, therefore, improve milk quality.

INDEX TERMS: *Pseudomonas* spp., ecology, contamination, bulk tanks, dairy farms, Amplified Fragment Length Polymorphism technique, AFLP, milk, food microbiology, psychrotolerant.

RESUMO.- [***Pseudomonas* spp.: fontes de contaminação em tanques de expansão em fazendas leiteiras.**] Este estudo se propôs a isolar *Pseudomonas* spp. durante o processo de ordenha em dez fazendas com sistemas manuais e mecanizados, durante as estações seca e chuvosa, além de avaliar a homologia do DNA e seus padrões de distribuição entre os isolados, a fim de se determinar as principais fontes de contaminação do leite. Cento e sessenta e sete isolados de *Pseudomonas* spp. foram obtidos a partir de amostras de água, mãos de ordenhadores, tetos, teteiras, tanques de resfriamento e leite cru armazenado, sendo 85 e 82 pontos de amostragem em fazendas com sistemas de ordenha manual e mecânico, respectivamente. Diferença estatisticamente significativa foi encontrada entre os isolados observados entre a superfície dos tetos antes e após o pré-*dipping* (p=0,02), mas nenhuma diferença foi encontrada entre sistemas de ordenha ou estações (p>0,05). A possibilidade do mesmo padrão de *Pseudomonas* spp. estar distribuído em diferentes fazendas ou estações foi avaliada

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pela técnica de Polimorfismo do Tamanho de Fragmento Amplificado (AFLP). As mãos de ordenhadores, superfície dos tetos das vacas, teteiras e tanques de resfriamento foram associados com a contaminação do leite cru, demonstrando que independente do tipo de ordenha e estação, a higiene adequada de equipamentos, utensílios e mãos dos ordenhadores é essencial para evitar contaminação do leite, e consequentemente aumentar sua qualidade.

TERMOS DE INDEXAÇÃO: *Pseudomonas* spp., ecologia, contaminação, tanques de expansão, Polimorfismo do Tamanho de Fragmento Amplificado, fazendas leiteiras, leite, microbiologia alimentar, psicrotolerante.

INTRODUCTION

Presence of extracellular enzymes, mainly heat-stable lipases and proteases produced by *Pseudomonas* spp. during refrigeration of raw milk in dairy farms, causes spoilage of dairy products. These microorganisms can contaminate refrigerated raw milk and produce spoilage metabolites (spoilage activity) even at 4ºC (Dogan & Boor 2003, Arcuri et al. 2008).

Several bacteria genera have been described as psychrotolerant microorganisms and *Pseudomonas* is the genus of most technological relevance (Hantsis-Zacharov & Halpern 2007). Since psychrotolerant microorganisms are commonly found in natural environment, dairy products can be contaminated by contact with water, inner surface of bulk tanks during storage of refrigerated raw milk, surface of cows' teats and equipment used throughout the milking process (Leriche et al. 2004, Fagundes et al. 2006, Teh et al. 2011). Therefore, the adoption of hygienic measures during milking process is necessary in order to reduce contamination with psychrotolerant microorganisms (Elmoslemany et al. 2010). So, the performance of molecular studies in order to evaluate the main contamination sources of raw milk with *Pseudomonas* spp. are required to understand which prophylactic measures are required to improve milk quality in Brazilian dairy farms.

Pseudomonas species have been isolated from different dairy products, such as cheeses, refrigerated raw milk, and pasteurized milk, while the presence of proteases has been described in ultra-high temperature (UHT) treated milk (Martins et al. 2006, Arslan et al. 2011, Beena et al. 2011, Chen et al. 2011). These enzymes are responsible for reducing dairy products shelf-life and destabilizing UHT milk (He et al. 2009, Baglinière et al. 2012). *Pseudomonas* spp. isolates also have high genetic diversity, different spoilage potential and antimicrobial resistance (Dogan & Boor 2003, Martins et al. 2006, Marchand et al. 2009a,b, Marques et al. 2012). Therefore, the use of molecular techniques helps to describe the diversity of these milk contaminants (Ercolini et al. 2009).

So the aim of this study was to isolate *Pseudomonas* spp. throughout milking process in dairy farms with manual and mechanical milking systems, during rainy and dry seasons, as well as evaluating DNA homology between isolates in order to verify the most important sources of contamination of milk by these microorganism and their patterns of distribution.

MATERIALS AND METHODS

Farms characterization. This study was performed in ten dairy farms located at Pirassununga, state of São Paulo, Brazil, belonging to the Rural Regional of the Development Office (EDR) Limeira/SP, during dry and rainy seasons of 2014 and 2015. During the study period, the average rainfall in the rainy and dry seasons was 85.20 and 21.85 mm, respectively (USP 2015). Cattle population size in these farms was from 10 up to 40 cross-breed cows on lactation and milk production ranging from 300 to 1.000 liters/daily. Milking was performed manually in five farms (A, C, D, I, J), while milking was mechanical in the other five properties (B, E, F, G, H). All farms had expansion tanks. Pre-dipping was performed using commercial products containing 2% of chlorine solutions.

Sampling sites. A pool of swabs, water and milk were sampled from ten different sites during milking process (Table 1).

***Pseudomonas* spp. isolation.** Pools of swabs were placed inside tubes containing 5mL of 0.1% peptone water. A set of 500mL of water and milk samples were collected in sterile glass flasks. Samples were kept in a cool box with recyclable ice until the analysis moment. *Pseudomonas* spp. enumeration was performed according to Cousin & Bramley (1981). For this purpose, dilutions of the samples were prepared by transferring aseptically 25mL of the sample to an Erlenmeyer containing 225mL of 0.1% sterile peptone water (10^{-1} dilution). Tenfold dilution until a 10^{-5} dilution were prepared from the same diluents. Subsequently, 0.1mL of the sample was spread with a Drigalski handle on plates containing supplemented (CFC-SR103-Oxoid) *Pseudomonas* Agar Base (CM 559, Oxoid). Plates were incubated at 28ºC for 48 hours and colonies were counted with a colony counter and multiplied by the dilution factor. Isolates were stored in BHI broth with glycerol 50% and kept frozen at -80ºC until the DNA extraction procedure. *Pseudomonas* spp. isolates used in this study are the same ones from Capodifoglio et al. (2016), which focused on evaluating differences on *Pseudomonas* spp. counts and the spoilage potential from isolates during dry and rainy seasons. So, the samples sites were considered just as positive (1) or negative (0) for *Pseudomonas* spp. presence in this paper.

Statistical analyses. All sample points evaluated during dry and rainy seasons in the same farm were considered as pair wise. Also, the following sampling points (milker's hands before and after milking; teats' surface before and after pre-dipping; and teat cups before and after milking) were considered as pair wise. The evaluation among *Pseudomonas* spp. isolation in sampling sites during dry and rainy seasons and among farms with manual or

Table 1. Different sampling points in ten dairy farms with manual and mechanical milking systems located in Pirassununga municipality, São Paulo State, Brazil, during the dry and rainy seasons of 2014-2015

Number	Sample point
1	Sample of cow's drinking water
2	Swab of milkers' hands before milking
3	Swab of milkers' hands after milking
4	Swab of the surface of cows' teats before pre-dipping
5	Swab of the surface of cows' teats after pre-dipping
6	Swab of internal surface of teat cups (mechanical milking) or milk bucket (manual milking) before milking
7	Swab of internal surface of teat cups (mechanical milking) or milk bucket (manual milking) after milking
8	Swab of internal surface of cooling tanks before milking
9	Milk from cooling tanks immediately after milking
10	Milk stored for 48 hours in the cooling tanks

mechanical milking was performed using binominal, chi-square or McNemar tests (p=0.05).

DNA extraction and quantification. DNA extraction was performed using DNAzol® (Invitrogen™) according to the manufacturer's instructions. A colony-forming unit for each sample site was collected and submitted to DNA extraction. Samples were subjected to DNA quantification by light absorbance using Nanodrop® spectrophotometry. All samples were stored at -80ºC until enzymatic digestion. The quantification of the isolated DNA ranged from 9.6 to 211.4ng/µl.

Digestion by restriction endonucleases. This research was based on AFLP Analysis System for Microorganisms (Life Technologies, Cat Numb. 11352-010 and 11352-018). The extracted genomic DNA was subjected to enzymatic digestion with two endonucleases (*EcoR* I and *Mse* I) simultaneously. *EcoR* I recognizes six pairs of nitrogenous bases (bp) (5'-GAATTC-3'), while *Mse* I recognizes a site of 4bp (5'-TTAA-3').Each sample was adjusted to 60ng/µL and incubated at 37ºC for two hours with endonucleases solution (*EcoR*I / *Mse* I 1.25 U/µL + buffer solution + ultrapure water). Enzymes were denatured at 70ºC for fifteen minutes.

Adapters' ligation. The digested DNA was subjected to ligation by EcoR I and Mse I adapters in order to generate the target DNA for amplification. Each sample received adapters' solution (adapters + T4 DNA ligase 1U/µL) and was incubated at room temperature (18 to 22ºC) for two hours. Afterwards, samples were diluted (1:10) in Tris-EDTA (TE) solution and stored at -20ºC until amplification.

Pre-amplification reaction. Pre-amplification reaction was performed with primers E-0 (5'-GACTGCGTACCAATTC-3') and M-0 (5'-GATGAGTCCTGAGTAA-3'), in order to allow a selective amplification, resulting in cleaner fingerprints.

Selective amplification reaction for *Pseudomonas* spp. The selective amplification reaction was performed with two different pairs of recommended primers for *Pseudomonas* spp. analysis: E-C (5'-GACTGCGTACCAATTCC-3') and M-A (5'- GATGAGTCCTGAGTAAA-3'); and E-C and M-G (5'- GATGAGTCCTGAGTAAG-3'). The thermal cycling protocol used was based on touchdown PCR in order to increase specificity, sensitivity and yield. PCR is started at a very high annealing temperature to obtain optimal primer selectivity. In the following steps the annealing temperature is lowered gradually to a temperature are which efficient primer binding occurs. This temperature is then maintained for the rest of the PCR cycles (Korbie & Mattick, 2008).The annealing temperature began at 65ºC per one cycle followed for 12 cycles with a decrease of 0.7ºC per cycle (touchdown phase of 13 cycles). Then, 23 cycles at 56ºC were performed. This reaction was performed in a final volume of 20 µL, according to manufacturer's instructions.

Polyacrylamide Gel Electrophoresis. The products from selective amplification reaction were separated using electrophoretic migration in denaturating polyacrylamide gel at 6% (Novex® TBE-Urea Gels, 6%, 15 well, Invitrogen™). The samples were denatured at 70ºC for three minutes using Novex® TBE-Urea Sample Buffer (Invitrogen™). The standard molecular size 30 to 330 bp AFLP® DNA Ladder (Invitrogen™) was used. Electrophoresis was performed in X Cell Sure Lock® Mini-Cell (Invitrogen™) with Tris--Borato-EDTA (TBE) buffer using voltage between 150 and 200 V for about one hour.

Silver staining. The gel staining was performed using SilverXpress® Silver Staining Kit (Invitrogen™) according to manufacturer's instructions. It was possible to detect signal from 0.5ng of DNA fragments equal or larger than 50 bp.

AFLP electrophoretic migration pattern analysis. Images were analyzed using the software BioNumerics version 7.50 (AppliedMaths) after photographic documentation, and the images normalization was performed from the molecular size standard. The levels of similarity between the AFLP patterns were calculated using Dice's coefficient. A tolerance of 1% was accepted for correspondence among bands. The strains were grouped by UPGMA (Unweighted Pair Group Method of Arithmetic Means – hierarchical method) for dendrograms construction (Geornaras et al. 1999). The isolates were considered as correlated when the similarity among them was higher than 80%.

RESULTS AND DISCUSSION

A total of 167 isolates of *Pseudomonas* spp. were obtained from 200 samples in 10 dairy farms with manual and mechanical milking systems, during the dry and rainy seasons, as shown in Table 2.

Pseudomonas spp. were isolated from 100% of the cooling tank samples, 95% of samples collected from teat cups after milking and from empty bulk tanks, 90% of the samples from the surface of cows' teats before pre-dipping and milk samples stored for 48 hours, 85% of samples of

Table 2. *Pseudomonas* spp. isolated from different sampling points in ten dairy farms with manual and mechanical milking systems located in Pirassununga municipality, São Paulo State, Brazil, during the dry and rainy seasons of 2014-2015

	Sites	Farms																				Number of isolates
		Manual milking										Mechanical milking										
		A		C		D		I		J		B		E		F		G		H		
		D*	W**	D	W	D	W	D	W	D	W	D	W	D	W	D	W	D	W	D	W	
1	Water	1	1	1	1	1	1	1	1	1	0	1	0	1	0	1	0	1	1	0	0	14
2	Milkers' hands before milking	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	0	1	0	1	16
3	Milkers' hands after milking	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	0	1	1	0	16
4	Surface of cows' teats before pre-dipping	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	18
5	Surface of cows' teats after pre-dipping	1	0	1	1	0	0	0	0	0	0	1	1	1	1	1	1	1	0	0	0	10
6	Teat cups before milking	0	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	17
7	Teat cups after milking	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	19
8	Cooling tanks before milking	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	19
9	Milk from cooling tanks immediately after milking	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	20
10	Milk stored for 48 hours in the cooling tanks	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	18
	Total of isolates	9	9	10	10	9	9	3	9	9	8	6	9	10	9	10	9	8	9	5	7	167

* Dry season, ** Rainy season, 0 = negative, 1 = positive.

the teat cups before milking, 80% of the samples collected from the milkers' hands before and after milking, 70% of water samples and 50% of the samples taken from cows' teats after pre dipping. These results highlight *Pseudomonas* spp. widespread in dairy farms environment due high quantity of positive samples.

Also, these results demonstrate that good hygienic practices are necessary to control *Pseudomonas* spp. contamination during the milking process and cold storage of raw milk in dairy farms, to improve the quality of milk used in manufacturing dairy products (Leitner et al. 2008, Vallin et al. 2009), since this bacterium is commonly reported as one of the main contaminants found in raw milk (Ercolini et al. 2009).

A total of 85 samples collected in properties with manual milking system were found to be positive for *Pseudomonas* spp., 40 collected during the dry season and 45 during the rainy season. On farms with mechanical milking, *Pseudomonas* spp. were isolated from 82 samples, of which 39 were taken during the dry season and 43 in the rainy season. This demonstrates a higher isolation rate in dairy farms with manual milking and during the rainy season. However, no significant differences ($p>0.05$) were observed on *Pseudomonas* spp. isolation among farms with mechanical or manual systems or among dry and rainy seasons. In our previous manuscript, a higher isolation rate of these bacteria was found in rainy season (Capodifoglio et al. 2016).

According to Marchand et al. (2009a), *Pseudomonas* spp. counts and lipolytic *Pseudomonas* spp. percentages in raw milk from Belgian dairy farms were higher during the winter months, which could be attributed to the different housing management practices. During winter, animals are kept in closed stalls, while in the summer months, cows are released on pastures. However, this difference was not observed in this research. In the present research the sampling sites were considered as positive or negative and it can explain the difference observed.

A significant difference ($p=0.02$) on *Pseudomonas* spp. isolation was observed among samples of the surface of cows' teats before and after pre-dipping using commercial products. This result highlights the importance on adopting pre-dipping before cow's milking in order to reduce *Pseudomonas* spp. contamination of dairy products and improve milk quality. Furthermore, no significant difference ($p>0.05$) was observed among milkers' hands before and after cow's milking and also among internal surface of teat cups (mechanical milking) or milk bucket (manual milking) before and after milking, demonstrating that contamination occurs during any step of milking process.

DNA amplification using the E-C and M-A or E-C and M-G primers were positive in 76 and 61 samples from farms with manual and mechanical milking system, respectively. Eleven different patterns were found and named using Romans numerals (I to XI). Only isolates that have shown correlation higher than 80% among samples are listed in Tables 3 and 4, and were used to establish the contamination sources of raw milk as shown in Figure 1.

Dairy products can be contaminated with a set of microorganisms by contact with water, inner surface of bulk tanks during storage of refrigerated raw milk and surface of cows' teats and equipment used throughout the milking process (Leriche et al. 2004, Fagundes et al. 2006, Teh et al. 2011), however, no correlation was observed among isolates obtained from water and raw milk in our study, highlighting that water is not one of the most important *Pseudomonas* species contamination sources for raw milk in the studied farms.

For samples collected during the rainy season in farm B, raw milk isolates of *Pseudomonas* spp. were similar to those found on the surface of cows' teats after pre-dipping (V) and on the cooling tank wall (II). During dry season, *Pseudomonas* spp. isolates found in milk samples were associated to the isolates from teats surface after pre-dipping and teat cups after milking (I). These patterns indicate that failures in pre-dipping and inadequately cleaned and disinfected equipment could lead to contamination to the stored milk and reduce its shelf-life. It is known that microorganism detected in cow's teats has similarities with populations detected on raw milk in bulk tanks (Doyle et al. 2016). Another two patterns were detected (III and IV). They show correlation between milkers' hands after mi-

Table 3. *Pseudomonas* spp. isolates with a minimum of 80% of correlation with milk samples collected from the cooling tanks immediately after and 48 hours after milking in 10 dairy farms located in Pirassununga municipality, state of São Paulo, Brazil, during the rainy season of 2014-2015

Farm	Rainy season			
	Primer MA		Primer MG	
	Milk from cooling tanks immediately after milking	Milk stored for 48h in cooling tanks	Milk from cooling tanks immediately after milking	Milk stored for 48h in cooling tanks
Ama	Not clustered	Not clustered	Not clustered	X
Bme	Not clustered	cooling tank before milking	Not clustered	Teats surface after pre-dipping
Cma	Not clustered	Not clustered	Not clustered	Not clustered
Dma	Not clustered	X	X	X
Eme	Not clustered	X	Not clustered	Not clustered
Fme	X	X	X	X
Gme	Not clustered	Not clustered	Not clustered	X
Hme	Not clustered	Not clustered	X	X
Ima	X	X	X	X
Jma	Not clustered	Not clustered	Not clustered	Not clustered

ma = manual milking system, me = mechanical milking system, X = no amplification or isolation.

Table 4. *Pseudomonas* spp. isolates with a minimum of 80% of correlation with milk samples collected from the cooling tanks immediately after and 48 hours after milking in 10 dairy farms located in Pirassununga municipality, state of São Paulo, Brazil, during the dry season of 2014-2015

Farm	Dry season			
	Primer MA		Primer MG	
	Milk from cooling tanks immediately after milking	Milk stored for 48h in cooling tanks	Milk from cooling tanks immediately after milking	Milk stored for 48h in cooling tanks
Ama	X	Not clustered	X	X
Bme	Not clustered	Not clustered	Teats surface after pre-dipping and teat cups after milking	Teats surface after pre-dipping, and teat cups after milking
Cma	Milkers' hands after milking, teats surface before pre-dipping	Bucket inner surface before milking	N Not clustered	Not clustered
Dma	Not clustered	X	X	X
Eme	Not clustered	X	X	Not clustered
Fme	X	X	X	X
Gme	Cooling tank before milking	Cooling tank before milking	X	X
Hme	Not clustered	X	Not clustered	X
Ima	X	X	Not clustered	X
Jma	Not clustered	Not clustered	X	X

ma = manual milking system, me = mechanical milking system, X = no amplification or isolation.

lking, cows' teats before milking and inner surface of teat cups, but apparently this contamination didn't reach the stored milk. Yet, it is important to notice that isolates from milkers' hands after milking and inner surface of teat cups after milking have shown 100% identity.

In dairy farm C, relationships between contaminated milk samples and isolates from milkers' hands after milking and surface of cows' teats before pre-dipping (VII and VIII), and inner surface of the bucket before milking (VI) were observed during dry season. This highlights the need for reinforcing hygienic practices during all stages of milking process to avoid *Pseudomonas* spp. contamination in raw milk (Fagundes et al. 2006).

When evaluating samples obtained from farm G, it was observed that surface of the cooling tank was the main source of contamination for milk produced during the dry season (IX). This finding is interesting because highlights the role of tanks surface to milk contamination, which commonly is attributed to the environment where cows are maintained during rearing (Doyle et al. 2016). Another interesting fact is that, during rainy season, isolates from surface of cows' teats before pre-dipping and teat cups before milking were 100% identical (X), but genetically distant of those were contaminating the stored raw milk. In this case, the correlation between cooling tank and stored milk was not taken in account, but, there was almost 80% of similarity, demonstrating association with the results from rainy season.

Farm J presented a pattern between milk from cooling tanks immediately after milking and milk stored for 48 hours during dry season (XI), but it was not possible to trace the origin of this contamination. Nevertheless, during rainy season, a correlation was almost established between stored milk and inner surface of cooling tank before milking, but it didn't reach 80% of similarity.

Tables 3 and 4 show that the milkers' hands and surface of cows' teats before pre-dipping are important sources of contamination in dairy farms with manual milking system, while in farms with mechanical milking, the surface of cows' teats after pre-dipping, teat cups and cooling tanks also contribute to with *Pseudomonas* spp. contamination. For milk samples collected directly from the cooling tanks (immediately after and 48 hours after milking), the incidence of contamination reached almost 100%, regardless of the type of milking system. Also, it was demonstrated the possibility of occurrence of *Pseudomonas* spp. contamination from different sources, demonstrating the need of adoption of hygienic management practices to avoid the dissemination of this bacteria. Furthermore, the formation of biofilms in the cooling tanks (Teh et al. 2011) also can be related to milk contamination by these microorganisms. Bacteria detected on farms can survive up to the receivement of milk in dairy industries causing technological failures (Almeida et al. 2017), i.e, bitterness, particle formation, creaming, sediment formation and gelation (Stoeckel et al. 2016).

As shown by Silva et al. (2011), when studying psychrotolerant bacteria (including *Pseudomonas* spp.), milkers' hands are one of the main sources of milk contamination in properties with manual milking. In this study, the importance of water, milkers' hands, surface of cows' teats, teat cups and cooling tanks as sources of contamination of raw milk with *Pseudomonas* spp. was highlighted. Molecular characterization of milk contaminants and a thorough understanding of the ecological and epidemiological aspects of *Pseudomonas* spp. are critical to establishing the most effective preventive measures (Ercolini et al. 2009) and to improving the quality of dairy products, since these microorganisms produce heat-resistant proteases, which cause technological failures that affect the processing steps during dairy products manufacturing (Marchand et al. 2009b). It is interesting to compare *Pseudomonas* spp. contamination sources in milk with other bacteria as *Bacillus cereus*, a important foodborne and spoilage microorganism in dairy production chain, which commonly is found on soil (Kumari & Sarkar 2016). According to Christiansson et al. (1999), the milking equipment did not contribute to milk's contamination, however, we highlighted the importance of them in *Pseudomonas* spp. contamination. Total counts

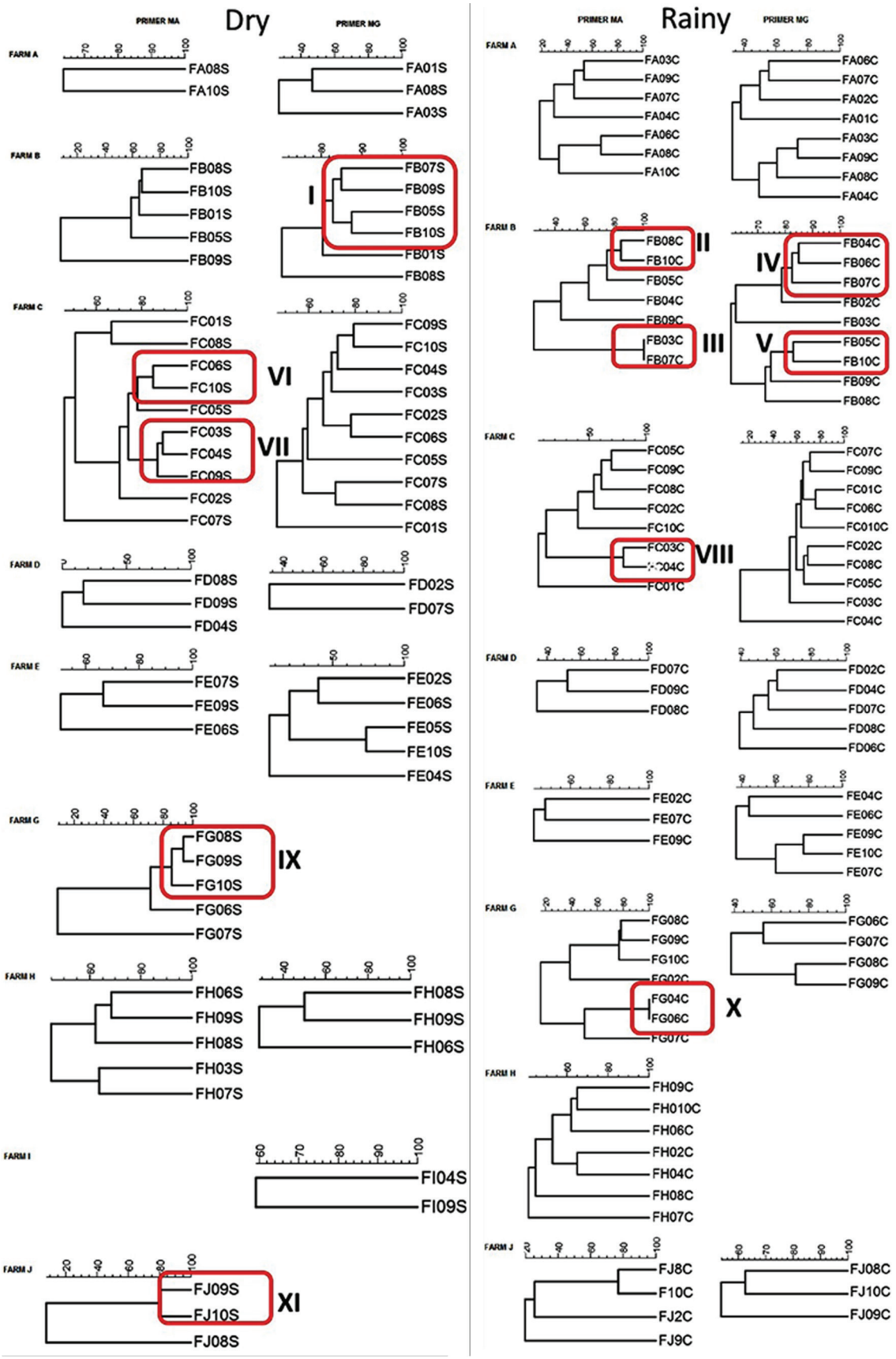

Fig.1. Dendrograms demonstrating genetic similarity between *Pseudomonas* spp. strains isolated at different sites in ten dairy farms during dry and rainy seasons of 2014-2015. (Subtitle: Farm (F), Farm letter (A, B, C, D, E, F, G, H, I, J), Site number (01, 02, 03, 04, 05, 06, 07, 08, 09 and 10), Dry season (S) or Rainy season (C). Romans numerals (I to XI) represent similarity patterns.)

of *Pseudomonas* spp. can be reduced by adopting proper hygiene practices during the milking process and storage of refrigerated raw milk (Fagundes et al., 2006, Silva et al. 2011), emphasizing the need for improvements in the dairy farms evaluated in this study. Since *Pseudomonas* spp. can even affect the processing of ultra-high temperature treated dairy products (Chen et al. 2011, Baglinière et al. 2012), good hygienic measures can contribute to the quality of dairy products and industrial productivity of the dairy industry.

To reduce the count of *Pseudomonas* spp. in milk, milking equipment and utensils should be cleaned with water of good quality, and disinfected immediately after use (FAO & WHO 1997). Pre-dipping should be properly performed (appropriate disinfectant solutions, concentration and period of action), once *Pseudomonas* spp. is able to survive in disinfectant solutions, such as those based on chlorhexidine and quaternary ammonium, (Nickerson 2001). In our study, pre-dipping was able on reducing *Pseudomonas* spp. detection on teats surface, stressing the need of adoption of this practice during milking, to improve the quality for raw milk and dairy products. Furthermore, the adoption of systems using continuos N_2 gas-flushing of raw milk can reduce *Pseudomonas* spp. growth (Gschwendtner et al. 2016).

It has been demonstrated that periodic and effective cleaning of cooling tanks is crucial, since these are a major source of contamination by *Pseudomonas* spp. The presence of bacterial colonies in bulk tanks may not only represent an important a source of contamination by viable cells of *Pseudomonas* spp., but also the presence of heat stable enzymes produced by these microorganisms, that can be detrimental to the quality of milk and dairy products (Teh et al. 2011). Proper functioning of cooling tanks should also be of concern in order to rapidly cool the milk and prevent bacterial multiplication (Chye et al. 2004).

CONCLUSIONS

Pseudomonas spp. was isolated from all milk samples and from all sampling points, so the dissemination of *Pseudomonas* spp. in dairy farm environment was demonstrated.

Milkers' hands, surface of cows' teats, teat cups and cooling tanks were associated with raw milk contamination with *Pseudomonas* spp. on farms with manual and mechanical milking system, showing that regardless of the type of milking system and season, proper hygiene procedures of equipment, utensils and workers' hands are essential to avoid contamination of raw milk.

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