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Occurrence of *Listeria monocytogenes*, total coliforms, *Escherichia coli*, and production and storage processes of raw milk from dairy farms in the state of São Paulo, Brazil

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In the present study, the occurrence of *Listeria monocytogenes*, total coliforms and *Escherichia coli* in refrigerated raw milk from 75 dairy farms distributed in three regions (São Carlos- A, Pirassununga- B, and Piracicaba- C) of São Paulo State, Brazil was assessed. The production and storage conditions as well as milking procedures of raw milk were also evaluated. The analysis of *L. monocytogenes* was performed according to the method established by the Food and Drug Administration (FDA). The Official Method was used for the analysis of coliforms using the SimPlate system. *L. monocytogenes* was not isolated from raw milk samples (n=286), although *Listeria innocua* has been isolated in the milking environment. Total coliforms counts above 10^3 MPN/mL were found in 86% (n = 85), 75% (n = 71) and 72% (n = 66) of samples from regions A, B and C, respectively. *E. coli* was found in 66% of samples in region A, 65% in region B and 46% in region C. Of the 75 farms surveyed, 77.3% showed inadequate conditions for milk production as well as insanitary milking equipment and utensils which certainly compromises the quality of milk and dairy products.

Key words: *Listeria, Escherichia coli,* milk quality, raw milk, microbiological quality.

INTRODUCTION

Sanitary factors at milking and of equipment, as well as the use of skilled workers are essential to produce milk hygienically and with good microbiological quality, which is required for the technology used in milk and milk products (Citadin et al., 2009). In Brazil, the regulations adopted by the Ministry of Agriculture, Livestock and Supply in recent years have compelled milk producers to improve the microbiological quality of raw milk, especially by requiring the cooling of raw milk on the dairy farms. The first regulation, Normative Instruction (IN) 51 (Brazil, 2002), was updated in 2013 by IN62 (Brazil, 2013), and the detailed criteria for the production, identification,

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quality and rating of milk in Brazilian dairy farms were established. According to the IN51 and IN62, the raw milk need to be refrigerated in the farm in bulk tank milk or in jar immersed in chilled water.

To comply with the new regulations, several investments on techniques for dairy production were done in Brazil, which have contributed in the improvement of the microbiological quality of milk and increasing milk production in dairy farms, especially in intermediate and large scale operations. However, nearly 30% of the total milk produced in Brazil comes from small scale farms (up to 400 L/day) (Battaglini et al., 2013; Paixão, 2013). The microbiological quality of milk from these dairy farms is rather variable, mainly because of lower investments as compared to large scales farms, lack of educational programs regarding hygiene procedures during milking and storage, and lack of milk refrigeration during transportation to dairy plants (Fagundes et al., 2011; Paixão, 2013).

Coliforms are good indicators of the sanitary conditions of production and storage of milk. As they are typically found in environments of the milking, inadequate hygiene practices can result in high coliform counts in raw milk (Bramley and McKinnon, 1990). The most used microbiological indicator of fecal contamination is *Escherichia coli* (Roitman et al., 1988), although several pathogenic microorganisms can also contaminate raw milk, especially *Listeria monocytogenes* (Van Kessel et al., 2004). *L. monocytogenes* is an important human pathogen, mainly because of the severity of the disease, listeriosis, which results in high mortality rates. *L. monocytogenes* is usually destroyed by pasteurization of milk, but recontamination can occur along the milk production chain (Waak et al., 2002). In dairy plants, raw milk can be an important source of *L. monocytogenes* contamination. Moreover, factors related to milking hygiene conditions in dairy farms were significantly associated with the contamination of raw milk with *L. monocytogenes* (Sanaa et al., 1993). In Brazil, previous studies have reported the incidence of *L. monocytogenes* in milk and dairy products (Destro et al., 1991; Casarotti et al., 1994; Moura et al., 1993; Silva et al., 2003; Nero, 2005; Arcuri et al., 2006; Barancelli et al., 2014). However, there is little information on the raw milk quality and production characteristics indairy farms in Brazil, especially after regulations IN 51 (Brazil, 2002) and IN 62 (Brazil, 2013) have been enforced. Therefore, the aim of this study was to evaluate the presence of *L. monocytogenes,* total coliforms and *E. coli* in samples of raw milk and their relation to different milking practices in small and intermediate scale dairy farms in São Paulo Brazil.

MATERIALS AND METHODS

The experiment was conducted in dairy farms in the regions of São Carlos (A), Pirassununga (B) and Piracicaba (C) of the northeastern region of São Paulo State, Brazil, between October 2008 and September 2009. Seventy-five dairy farms were analyzed, 25 from each region and the farms were visited four times at intervals of approximately two months. Questionnaires were used to characterize the farms in terms of milking conditions and the raw milk storage system. They were applied on site and were based on current regulations (Brazil, 1997, 2002) and Spexoto (2003).

A total of 286 samples (500 mL) of refrigerated raw milk were collected from region A (N = 99), region B (N = 95) and region C (N = 92), directly from the tanks or drums, after homogenization with the aid of sterile ladles and placed in sterilized jars. Nine farms (three per region) were selected for milk collection with a Moore's strand (Lacen, 2000), which remained in the tank of raw milk for about 12 h before the procedure, performed in sterile packaging in order to increase the chance of isolation of *L. monocytogenes*. Before collection and after homogenization, the milk temperature was measured with a digital thermometer. From three selected farms (one per region), samples from the milking environment were collected including drains (N = 6), floor of the milking area (N = 3), liners (N = 10), floor of the cooling room (N=5), udders and teats surface of lactating cows (N = 18), silage (N = 2) and surface milk in the storage tank (N = 1). For the collection of environmental samples, sponges (Inlab) moistened in saline (0.85%) and peptone (0.1%) were used, added with neutralizing sanitizers: 0.01% sodium thiosulfate (Silva et al., 2003), 0.5% polysorbate (Tween 80) and 0.07% soybean lecithin (Evancho et al., 2002). After collection, the sponges were placed in bags with 60 ml of Listeria Enrichment Broth Buffered (BLEB) (Difco). The samples were transported in coolers with ice to the Laboratory of Hygiene and Dairy College of Agriculture "Luiz de Queiroz" (ESALQ) where they were analyzed.

Samples of raw milk and the Moore's strands and environmental samples (collected with sponges) were analyzed according to the methodology recommended by the Food and Drug Administration (Hitchins, 2003). For the isolation of *L. monocytogenes* in raw milk and the milking environment, 50 ml of milk was inoculated into 450 ml of BLEB. Swabs and the Moore's strands were inoculated with 225 mL BLEB. The sponges were homogenized in a Stomacher strands before incubation. The samples were incubated at 30°C/48 h. After 4 h from the start of incubation, acriflavine (10 mg/L) and nalidixic acid (40 mg/L) and cycloheximide (50 mg/L)were added. After incubation, the striation in the Oxford agar (Oxoid) and Listeria agar was carried out according to Ottaviani and Augustine(ALOA - AES Chemunex), which were incubated at 35°C/24-48 h and 37°C/24-48 h, respectively. Three characteristic colonies from each medium were purified on Trypticase Soy Agar (TSA) (Oxoid) with 0.6% yeast extract. For the biochemical confirmation of the suspect colonies, the analyses of catalase, Gram stain and characteristic motility at 25°C were performed, and the ApiListeria Kit (BioMérieux) was used to characterize the species. The strain of *L. mocytogenes* ATCC 7644 was used as a positive control. For the enumeration of total coliforms and *E.coli*, successive dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) of the milk in saline solution (0.85%) and peptone (0.1%) were prepared. The analyzes were performed on SimPlate CEC (BioControl Systems, Inc.) according to the Official Method 2005.03 (AOAC, 2005). Aliquots of raw milk and its dilutions were used for the analyses with the hatching plates of 32°C/24 h. The wells with a purple color were considered positive for total coliforms and those that were fluorescent under ultraviolet light (366 nm), positive for *E. coli*. The most probable number (MPN) was determined in the appropriate table and the result expressed as MPN/mL of milk. The statistical analysis was done by comparing the counts of coliforms and *E. coli* with the selected questionnaire items using multiple comparisons of means (Tukey test), adjusted for the level of significance ($p< 0.05$).

RESULTS AND DISCUSSION

Table 1 shows the daily milk production, type of milking

Table 1. Characteristics of milk production in farms in São Carlos (A), Pirassununga (B) and Piracicaba (C) regions, Brazil.

Characteristic	A n (%)	B n (%)	C n (%)
Daily milk production			
Up to 100 L	11(44)	1 (4)	10 (40)
>100-500 L	11(44)	18 (72)	14 (56)
>500-1000 L	0 (0)	4 (16)	1 (4)
>1000-3000 L	2 (8)	2 (8)	0 (0)
>3000-5000 L	1 (4)	0 (0)	0 (0)
Type of milking			
Mechanical - canalized	1 (40)	5 (20)	0 (0)
Mechanical - bucket at foot	12 (48)	12 (48)	15 (60)
Manual	12 (48)	8 (32)	10 (40)
Refrigeration system			
Bulk tank milk	11 (44)	22 (88)	22 (88)
Jar of milk immersed in chilled water	14 (56)	3 (12)	3 (12)

n: Number of farms (total number of farms studied: 25 in each region).

and refrigeration system of the 75 farms studied. The three regions had a predominance of small producers, with 86.6% (N = 65) producing up to 500 L/day. This characteristic of small-scale production is also typical in other regions in Brazil (Nero et al., 2005; Monteiro et al., 2007; Brito et al., 2004). In 45 farms (60%), the milk was obtained by mechanical milking devices, while 30 farms (40%) had manual milking. Regarding the refrigeration system, 55 farms (73%) used bulk milk tanks, and 25 farms (33%) used milk jars immersed in chilled water for cooling the raw milk. In a study conducted in Paranapanema, also in São Paulo state, Furlaneto et al. (2008) found a higher percentage of dairy farms with manual milking (77%). This practice is also widely used in other states in Brazil, such as Rio Grande do Sul, where Moraes et al. (2005) found 50% of 41 farms using manual milking, and in the Northeastern states, where 88% of 41 farms use manual milking, and only 24.4% used community bulk tanks (Monteiro et al., 2007). The community bulk tanks are used by a group of small producers, from different farms, to cool the raw milk in a unique place. This has been a valuable strategy adopted in Brazil to reduce costs, aiming to improve the price of milk for producers with increasing scale (Pereira and Magalhães, 2012).

Table 2 presents the milking practices in dairy farms from the three regions, indicating that most producers did not comply with basic and critical points to avoid the risk of milk contamination, such as washing and drying procedures of teats, and use of pre-and post-dipping. The appropriate handling of milking procedures is one of the most important strategies to ensure good quality of raw milk (Fonseca and Santos, 2000). Our results are in agreement with data reported in previous studies showing unsatisfactory production conditions in dairy farms in various regions of Brazil (Monteiro et al., 2007; Silva et al., 2008; Zegarra et al., 2007; Arcuri et al., 2006). In the present study, high temperatures of raw milk stored in the farms were reported, mainly in region B, with temperatures between 2-14°C, and even in cooling tanks, the temperatures reached 14°C. These data are consistent with the temperatures up to 14°C reported by Tebaldi et al. (2008) in cooling tanks in Minas Gerais State, Brazil.

L. monocytogenes was not isolated from the raw milk samples analyzed (n = 286). Importantly, in the current study, a higher number of samples of raw milk was analyzed, hence confirming the low prevalence or absence of *L. monocytogenes* in raw milk collected in dairy farms in Brazil, as observed previously by Casarotti et al. (1994) (n = 20); Nero (2005) (n = 240); Arcuri et al (2006) (n = 42) and Barancelli et al. (2014) (n = 16). In contrast, Moura et al. (1993) isolated *L. monocytogenes* from 9.5% of raw milk samples (n = 220) from São Paulo State. Moreover, highest prevalence rates were reported in raw milk collected from processing plants in Brazilian Northern states. Catão and Cebalos (2001) obtained 37.8% (n = 45) of positive samples, and Silva et al. (2003) found the pathogen in 16.7% (n = 6) of samples. Differences in the occurrence of the pathogen can be explained by the geographical distribution of the genus *Listeria* (Van Kessel et al., 2004). Furthermore, the detection of *L. monocytogenes* in raw milk can be difficult because of low numbers of bacteria and bacterial

Table 2. Milking practices (presence/absence) in farms in São Carlos (A), Pirassununga (B) and Piracicaba (C) regions, Brazil.

Milking practice	A		B		C	
	Presence n (%)	Absence n (%)	Presence n (%)	Absence n (%)	Presence n (%)	Absence n (%)
Use of proper uniform	2 (8)	23(88)	4 (16)	21(84)	1(4)	24(96)
Any washing of the teats	25(100)	0 (0)	24(96)	1(4)	23(92)	2 (8)
Use of pre-dipping	7 (26)	18(72)	12(48)	13(52)	14(56)	11(44)
Any drying of the teats	24 (96)	1 (4)	20(80)	5(20)	22(88)	3(12)
Use of paper towel	4 (16)	21(84)	9(36)	16(64)	9(36)	16(64)
Use of post-dipping	5 (20)	20(80)	9 (36)	16(64)	16(64)	9(36)
Use of gloves for milking	3 (12)	22(88)	3(12)	22(88)	0(0)	25(100)
Paved floor in corral waiting area	15 (60)	10(40)	13(52)	12(48)	21(84)	4 (16)
Cleaning of corral waiting area	5 (20)	20(80)	4(16)	21(84)	1(4)	24(96)
Flies in the waiting area	21 (84)	4 (16)	22(88)	3(12)	24(96)	1(4)
Paved floor in milking parlor	17 (68)	8 (32)	17(68)	8(32)	23(92)	2(8)
Cleaning of waiting room	5 (20)	20(80)	4(16)	21(84)	1(4)	24(96)
Washing of equipment/ utensils in hot water	7 (26)	18(72)	6(24)	19(76)	3(12)	2(88)
Use of sanitizing rinse in equipment/utensils	7 (26)	18(72)	5(20)	20(20)	13(52)	12(48)

n: Number of farms (total number of farms studied: 25 in each region).

Table 3. Distribution of total coliforms and *Escherichia coli* in samples of raw milk in São Carlos (A), Pirassununga (B) and Piracicaba (C) regions, Brazil.

Range (MPN/mL)	A n (%)	B n (%)	C n (%)
Total coliforms			
$<10^2$	3 (3)	8 (9)	7 (7)
10^2-10^3	11 (11)	17 (18)	19 (19)
$>10^3$-10^4	13 (13)	15 (16)	19 (19)
$>10^4$-10^5	17 (17)	23 (24)	21 (21)
$>10^5$	55 (56)	33 (34)	26 (26)
Escherichia coli			
<1	35(34)	33 (3)	48 (52)
>1-10	17(17)	17 (18)	17 (18)
>10-10^2	22 (24)	27 (28)	17 (18)
$>10^2$-10^3	9 (9)	5 (5)	4 (4)
$>10^3$	16 (16)	13 (14)	6 (6)

n: Number of samples. Total number of samples analyzed in each region: 99 (A), 95 (B) and 92 (C).

microflora competition (Meyer-Broseta et al., 2003). The uneven distribution of bacteria in large volumes, as in raw milk tanks, can also hinder their isolation. Thus, the absence of *L. monocytogenes* in samples does not mean that the pathogen was not present in the batches of raw milk analyzed. Regarding the milking environment, *L. innocua* was isolated in two points: the floor drain and a farm milking room in region C, representing 4.4% of the environmental samples.

The total coliforms and *E. coli* counts in samples collected in the three regions studied are shown in Table 3. In region A, 72 milk samples (73%) presented $>10^4$ MPN/mL of coliforms, hence indicating poor hygienic conditions of raw milk, considering that coliform bacteria are not part of the native micro flora of milk (Roitman et al., 1988). High coliforms counts ($>10^4$MPN/mL) were also found in 56 (68%) and 47 (47%) raw milk samples from regions B and C, respectively. *E. coli* was found in 64 (65%), 62 (65%) and 44 (46%) samples from regions A, B and C, respectively, indicating risk to human health, fecal contamination and possible presence of intestinal pathogens. In the United States, Van Kessel et al. (2004) found a higher percentage of raw milk samples contaminated with fecal coliforms (93% of 859 samples), and approximately 40% of 419 samples with populations between 10-10^2colony forming units/mL (CFU/mL). In the present study, *E. coli* counts higher than 10^2 MPN/mL were found in 25 (25%), 18 (19%) and 10 (10%) samples from regions A, B and C, respectively. Also, a significant difference ($p< 0.05$) between coliform counts in raw milk from the same farm in different sampling times was observed, indicating that there is no standardization or consistency in milking practices. The total coliforms counts in raw milk were not different ($p>0.05$) in farms with mechanical or manual milking and teat washing, which is similar to the results described by Moraes et al. (2005) and Gottardi et al. (2008). However, the average counts of total coliforms was significantly lower ($p<0.05$) for the farms that performed procedures for pre and post-dipping, had paved floor in the milking parlor, and had milk tank equipped with a cooling system.The coliform

Table 4. Total coliforms and *Escherichia coli* in samples of raw milk, according to the daily milk production of dairy farms in São Carlos, Pirassununga and Piracicaba regions, Brazil.

Milk production (L/day)	N	Total coliforms[1] (MPN/mL)	*Escherichia coli*[1] (MPN/mL)
<100	64	$3.9x10^4 \pm 0.64 \times 10^4$	$1.3 \times 10 \pm 0.35 \times 10$
500-1,000	6	$2.2 \times 10^5 \pm 1.95 \times 10^5$	$7.2 \times 10^2 \pm 2.3 \times 10^2$
1,000-3,000	4	$1.5 \times 10^4 \pm 0.83 \times 10^4$	$6.9x10 \pm 0.1 \times 10$
3,000-5,000	1	$3.9x10^3 \pm 0.1 \times 10^3$	$1.2 \times 10^2 \pm 0.1 \times 10^2$

[1]Results are expressed as mean ± standard deviation; N: Number of farms (total number of farms studied: 25).

Table 5. Total coliforms and *Escherichia coli* in samples of raw milk, according to the overall hygienic category of dairy farms from São Carlos, Pirassununga and Piracicaba regions, Brazil.

Hygienic category	N	Total coliforms[1] (MPN/mL)	*Escherichia coli* (MPN/mL)
1	2	$5.6 \times 10^3 \pm 0.2 \times 10^3$	$1.7 \times 10^2 \pm 0.63 \times 10$
2	15	$1.1 \times 10^5 \pm 1.1 \times 10^5$	$3.8 \times 10^2 \pm 0.75 \times 10$
3	58	$4.9 \times 10^4 \pm 1.1 \times 10^4$	$2.0 \times 10^2 \pm 1.10 \times 10$
Total	75	$1.4 \times 10^4 \pm 1.1 \times 10^4$	$2.5 \times 10^2 \pm 0.83 \times 10$

[1]Results are expressed as mean ± standard deviation. N: Number of farms (total number of farms studied: 25).

counts found in the present study were lower than those reported by Moraes et al. (2005), who found nearly 100% of raw milk samples from 42 farms in the state of Rio Grande do Sul with counts ranging from $2.3x10^3$ to $3.0x10^5$CFU/mL. Tebaldi et al. (2008) analyzed milk from 16 farms in the state of Minas Gerais, and found total coliforms in all samples with counts around 10^5 MPN/mL.

Table 4 presents the distribution of total coliforms and *E. coli* in samples of raw milk according to the daily milk production of dairy farms studied. The counts of total coliforms were higher in raw milk from producers with less than 1,000 L/day, especially in the 500-1,000 L/day category. However, *E. coli* counts were higher in dairy farms from 500-1,000 and 3,000-5,000 L/day categories. The reasons for the differences are difficult to access at this time. The hygienic procedures may be easily implemented in larger than in smaller operations, as a result of greater investments, but the higher number of lactating cows in large dairy farms also requires more equipment to sanitize and extend milking procedures, which can facilitate the gaps and opportunities for contamination of milk. Regardless of the daily milk production, results of this trial indicate the need for effective educational programs on good agricultural practices addressed to dairy farms in Brazil, in order to prevent the contamination of raw milk.

The data presented in Table 2 on the milking practices were used for classification of the 75 dairy farms in three categories in the overall hygienic conditions items, as follows: category 3 (n = 58, 77.3%), which comprised farms with poor conditions of production and hygiene of equipment and installations; category 2 (n =15, 20%), formed by farms with fair, intermediate conditions; and category 1, which had only 1 (2.7%) farm showing good hygienic conditions of milk production (Table 5). As expected, raw milk from dairy farms in the categories 2 and 3 showed higher mean counts of total coliforms than category 1. Of the 75 farms, only 2 (one from category 1 and one from category 2) fully met the IN 51/62 guidelines and had milk with better microbiological quality than the other 73 farms. However, *E. coli* counts were similar among the categories, indicating that fecal contamination of raw milk is not completely related to the environmental contamination during milking procedures in dairy farms.

The high coliforms and *E. coli* counts obtained indicate difficulties and/or lack of knowledge of the farmers to comply with the regulations of IN 51 (Brazil, 2002) and IN 62 (Brazil, 2013) as adopted in Brazil for raw milk. Although *L. monocytogenes* was not detected in raw milk samples, *Listeria innocua* was isolated in the milking environment, indicating that this site may be an important source of *Listeria* spp. Therefore, educational programs should be done to improve milk quality, especially in small and intermediate scale dairy farms.

Conflict of Interest

The authors have not declared any conflict of interest.

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