Detection of Mycobacterium avium subsp. paratuberculosis and other mycobacteria in retail milk and dairy products in Argentina

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RESUMEN

Mycobacterium avium subesp. paratuberculosis (MAP) es el agente etiológico de la paratuberculosis o la enfermedad de Johne (JD), una enteritis crónica que afecta al ganado vacuno y otras especies animales que se la vincula con la enfermedad de Crohn, una afección intestinal inflamatoria crónica en humanos. MAP podría resistir las condiciones de pasteurización y se lo aisló de la leche al por menor en varios países. Los objetivos fueron identificar MAP y otras micobacterias en leche y productos lácteos al por menor en Argentina y evaluar la calidad de estos a través del conteo de bacterias aerobias totales mesófilas (TAB). Se analizaron 384 muestras de leche de venta minorista durante 24 meses. Todas las muestras resultaron negativas en cuanto al crecimiento de micobacterias. Sin embargo, el 1.56% de las muestras dieron positivas en la identificación de MAP por IS900-PCR. El recuento de TAB superó los límites establecidos por el Código Alimentario Argentino (CAA) en el 4.95% de las muestras. No se detectaron micobacterias distintas de MAP ni por cultivo ni por PCR. La PCR positiva en MAP resultante de muestras de leche al por menor indicaría que podrían provenir de granjas lecheras con JD y sugiere que la leche pasteurizada o los productos lácteos no son fuentes significativas de exposición humana a MAP en Argentina. Algunas muestras de leche excedieron los límites establecidos por la AAC para los recuentos de TAB, lo cual indica que la leche comercializada podría procesarse y almacenarse incorrectamente.

Palabras clave: *MAP, micobacterias, control de calidad, leche, lácteos.*

ABSTRACT

Mycobacterium avium subsp. paratuberculosis (MAP) is the etiologic agent of paratuberculosis or Johne's disease (JD), a chronic enteritis disease that affects cattle and other animal species and has been linked to Crohn's disease, a chronic inflammatory bowel disease in humans. MAP could resist pasteurization conditions and has been isolated from retail milk in several countries. The aims were to identify MAP and other mycobacteria in retail milk and dairy products in Argentina and to assess the product quality through Mesophilic Total Aerobic Bacteria (TAB) count. Three hundred and eighty-four samples of retail milk were tested for 24 months. All samples were negative for mycobacteria growth. However, 1.56% of the samples were positive for MAP identification by IS900-PCR. The TAB count was higher than the limits established by the Alimentary Argentinian Code (AAC) in 4.95% of the samples. Mycobacteria other than MAP were not detected either by culture or PCR. The MAP positive PCR from retail milk samples would indicate that they could come from dairy farms with JD and suggests that pasteurized milk or dairy products are not significant sources of human exposure to MAP in Argentina. Some milk samples exceeded the limits established by the AAC for TAB counts, indicating that commercialized milk could be processed and stored incorrectly.

Keywords: MAP, mycobacteria, quality control, milk, dairy products.

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INTRODUCTION

Mycobacterium avium subsp. paratuberculosis (MAP) is the causative agent of Johne's disease (JD) in domestic and wild animals. In cows, it causes chronic enteritis, diarrhea, weight loss, and progressive emaciation or death. MAP transmission occurs by multiple routes, including fecal-oral, intrauterine, and through milk or colostrum. Infected cattle can shed bacteria in their feces and milk and spread the infection (Corbett *et al*., 2018; Wolf *et al*., 2014). MAP has also been linked to human Crohn's disease, a systemic disorder that causes mainly a chronic inflammation of the intestine (Bull *et al*., 2003; Feller *et al*., 2007; Rhodes *et al*., 2014). Many researchers have shown that the animals with JD can excrete live MAP in milk (McAloon *et al*., 2016), being resistant to thermal processes (Grant, 2006; Slana *et al*. 2008). MAP has been isolated from pasteurized commercial milk in Argentina, Brazil, India, Italy, United Kingdom, United States and Czech Republic between 0.3% - 67% of milk samples; (Grant *et al*., 2002a; Grant *et al*., 2002b; Ellingson *et al*., 2005; Ayele *et al*., 2005; Shankar *et al*., 2010; Paolicchi *et al*., 2012; Carvalho et al., 2012; Serraino *et al*., 2017, Gerrard *et al*., 2018). Other researchers identified MAP in commercial dairy products like cheeses, yogurt and powder milk by culture and PCR (Hruska *et al*., 2005; Botsaris *et al*., 2010; Shankar *et al*., 2010). The survival of MAP to heat treatments is related to the initial number of live bacteria in raw milk. MAP has been detected after high temperature short-time pasteurization (HTST), if the initial number of viable bacteria was greater than 1 x 105 CFU.mL-1 (Gao *et al*., 2002). This survival has also been linked to the MAP forming clumps. For this reason, combining pasteurization with homogenization increases the effectiveness of the heat treatment (Grant *et al*., 2005; Hammer *et al*., 2014).

The number of bacteria present in food products reflects the sanitary conditions of processing and allows determining the preservation and consumption time (Pinzón Fernández, 2006). Milk is a complete food for its nutritional characteristics, and an ideal growing medium for a great variety of microorganisms. In raw milk, microorganisms with different metabolic characteristics are found. The most important quality indicator group is called Mesophilic Total Aerobic Bacteria (TAB) and the concentrations of these bacteria are applied as a quality standard in raw milk (Morton, 2001). Furthermore, some outbreaks of foodborne diseases have been linked to the consumption of commercial milk processed with deficient thermal treatment, post-pasteurization contamination or incorrect storage conditions (Swai *et al*., 2011). In Argentina, there are no data about the TAB counts on samples of pasteurized milk from retail shops.

Based on previous data (Paolicchi *et al*., 2012) were MAP was identified using a small sample size,the aims of this study were to identify the presence of MAP and other mycobacteria in retail milk, yogurts and cheeses by culture and PCR and to assess the quality of commercial milk through TAB counting.

MATERIALS AND METHODS

Samples

Simple random and purposive sampling methods were followed to select the study samples. The sample size was determined based on the expected prevalence from the published data and the desired precision of 5% at 95% confidence level. The sample size was calculated using the standard formula described by Thrusfield *et al*., (2005). During 2013 and 2014. For 24 months, 384 commercial milk samples from the Mar y Sierras Basin, Argentina were evaluated. Twenty-four samples (3 replicates for each variable) were taken according to the following variables: commercial brand (first quality and second quality, based on relation price-quality criteria), thermal treatment (ultra pasteurized (UP) and ultra high temperature (UHT)), fat content (whole and skim) and seasons (autumn/ winter and spring /summer). Three packs of 1L of commercial milk from each category were randomly taken from retail points.

For 12 months, 24 yogurt samples and 24 soft cheeses samples from Argentina, were evaluated. One pack of 1L of commercial yogurt and 1 pack of soft cheese from each category were randomly taken from retail points. Three samples of each category were analyzed, considering the following variables: commercial brand (first quality and second quality), fat content (whole and skim) and seasons (autumn –winter and spring – summer). All samples were carried to the laboratory at 4°C until the analysis.

Culture

The samples were processed without chemical decontamination. From each pack, 150 mL of milk were taken and centrifuged (centrifuge Sorvall RC5CE, Newtown, USA) at 9,400 rpm for 15 min at 10°C. The fat layer and the pellet were processed. The pellet was suspended in 3mL of PBS. Four drops were inoculated in each of the following culture media: Herrold's egg yolk medium (HEYM), HEYM plus pyruvic acid (Sigma Chemical Co., St. Louis, Mo, USA.), HEYM plus antibiotics (vancomicin 0.01% w/v, amphotericin B 5% w/v, nalidixic acid 0.3% w/v y nistatin 0.01% w/v), Stonebrink and Löwenstein Jensen. The slopes were incubated at 37°C for 16 weeks and examined weekly for presence of MAP and mycobacteria colonies. Suspected colonies were analyzed microscopically using Ziehl Neelsen (ZN) stain (OIE, 2011).

PCR

DNA extraction by immunomagnetic beads (IMB): For identification of MAP by IS900-PCR 1 mL of the suspension obtained from each sample for culture was heated at 50°C during 15 min. Then each sample was centrifuged at 7,500 rpm at 4°C, during 15 min (Hermle, Z233MK-2, Germany). The milk fat and serum were discarded, and the precipitate was processed as follows: IMB coated with Goat Anti-Mouse IgG England, BioLabs, Inc., #S1431S, USA) were employed at 3.65 x 1010 IMB/mL, with adhesive capacity of 1 mg/5 µg of murine IgG. The suspension was homogenized by slow and constant agitation at 4°C during 1 h. In another tube, 10 µL of IMB mix (3.65 x 108) plus 10 µg/µL of polyclonal antibody - antiMAP was homogenized by slow and constant agitation at 4°C during 1 h. Then, the samples were collocated in a magnetic rack during 10 min. The complex IMB-Ac was obtained in the wall of the microtube, and the rest of the material was discarded. Afterwards, three washings with PBS were performed, separated with IMB and suspended in 100 µL of PBS.

DNA extraction by commercial kit: For Mycobacterium spp. detection by hsp65-PCR, 300 µL of the samples were taken. DNA was extracted using QIAamp™ DNA blood Mini Kit (Qiagen AG, Basel, Switzerland) following the manufacturer's in-

structions. For IS900 identification, the DNAs obtained were amplified as follow: the mix was prepared with 10 L of green buffer (Promega, USA), 4 L of dNTPs (2.5 mM each) (ABGene, USA), 0.25 mM of Primer dir C (GATCGGAACGTCGGCTGGTCAGG), 0.25 mM of Primer rev C (GATCGCCTTGTCATCGCTGCCG) (Collins *et al*., 1993), 0.3 L of Taq polymerase, 28.7 L of distilled water and 5 L of DNA sample, final volume 50 L. The samples were amplified by touch down PCR technique (Zumarraga *et al*., 2005): briefly, initial melting to 96°C during 3 min, followed by 10 cycles of de 96°C, 1 min; the annealing of primers at 72°C, 1 min; decreasing 1°C per cycle and extension at 72°C, 1 min. This step was followed by 30 cycles of 96°C, 1 min; 62°C, 1 min and 72°C 1 min. Finally, one cycle of 72°C, 8 min for final extension. For each set of PCRs, a positive control (DNA of MAP) and a negative control (distilled water) were added. The amplified product was subjected to electrophoresis in agarose gel (2% w/w) and the DNA was visualized in blue light transilluminator with Sybr Safe™. A sample was considered as positive if a 217bp amplified product was obtained.

For Mycobacterium spp. identification the sequence hsp65 was amplified using the same described mix but using 2 different primers: TB11 (ACCAACGATGGTGTGTCCAT) and TB12 (CTTGTCGAACCGCATACCCT) (Telenti *et al*., 1993). The samples were amplified as follow: 45 cycles of 96°C, 1 min; 56°C, 1 min and 72°C 1 min; and a final extension of 72°C, 8 min. For each set a positive control (DNA of mycobacteria) and negative control (sterile distilled water) were included. The amplified product was subjected to electrophoresis in agarose gel and the DNA was visualized in blue light transilluminator with Sybr Safe™. A sample was considered as positive if a 440bp amplified product was obtained.

TAB count

Each container was manually shaken and disinfected externally with 70% ethanol. One mL of each sample was taken to perform the TAB count according to the standard of International Dairy Federation FIL-IDF 100B-1991 (FIL-IDF, 1991). Serial dilutions of the samples were made (1:10, 1:100 y 1:1000) in peptone water (5% w/w) (Oxoid, England). Then, 1 mL of each dilution was spread onto 2 Petri plates where it was mixed with 15 mL of liquid Plate Count Agar (PCA, Oxoid, England) at 45°C. Once the agar had solidified, the plates were incubated for 48 ± 3 h at 37°C. The CFU counts was performed in those plates with 10 and 300 colonies. The average number of CFUs of both plates corresponding to the same dilution was multiplied by the inverse of the dilution factor to estimate the initial TAB CFU. According to Alimentary Argentinian Code (AAC), UP and UAT milk must meet the following requirements: TAB count less than 1000 CFU.mL-1 and less than 100 CFU.mL-1 (acceptable quality, followed by 7 days incubation at 35-37°C) of milk, respectively (articles 559tris and 560). Considering these limits, the samples were classified as "Not apt for consumption" or "Apt for consumption".

Identification of isolated bacteria

From PCA culture plates used for TAB counts, 3 bacterial colonies were randomly selected and purified on Columbia Blood Agar (Oxoid, England) for subsequent identification through biochemical testing.

Statistical analysis

For IS900-PCR results and TAB counts the data were analyzed using a model of multivariable logistic regression (Proc. LOGISTIC, SAS Studio v3.6, SAS Institute Inc., Cary, NC). The statistical analysis of the IS900-PCR and TAB counts was performed to evaluate the association of the different categories of the selected factors on these results. The response variable was positive or negative IS900-PCR and the presence/absence of TAB counts above the limits established by the AAC. The explicative variables were season (autumn – winter/ spring-summer), fat content (whole / skim), the brand (first /second) and the heat treatment (UP/UHT). The variables that showed a value p≤0.10 in the univariate analyses were included in a final multivariate model. The relative risk of exceeding the limits established by the AFC was estimated by calculating the odds ratio (OR) for each of the variables included in the model.

RESULTS

Detection of MAP and mycobacteria

All retail milk and milk products samples were negative for Mycobacterium spp. cultures. Using IS900-PCR for MAP detection, it was found that 6/384 (1.56%) samples of commercial milk were positive (table 1 and figure 1). The samples of commercial yogurts and cheeses were negative for IS900-PCR. All the samples of milk, yogurts and cheeses were negative for detection of hsp65-PCR gen for identification of *Mycobacterium* spp.

TAB count

Twenty-four milk samples (6.25%) were not apt for consumption exceeding the limits established by the AAC. Sixteen of these samples (4.17%) were UHT. Also, considering the totality of not apt for consumption, 23 (5.99%) samples corresponded to the second brand commercial milks. The greatest number of samples that exceeded the limits established by the AAC was taken during Spring – Summer (table 2 and figure 2).

Identification of bacteria

Colonies extracted from the plates of counts were identified as belonging to Flia. Enterobacteriaceae and the following genera: Staphylococcus sp., Flavobacterium sp., Aeromonas sp., Bacillus sp., Kurthia sp.

Statistical analysis

For IS900-PCR results no significant effect between variables was observed (p>0.10) (table 1 and figure 1). For TAB count, a significant effect of the time of year and commercial brands was observed (p<0.01). No significant effect of fat content or interactions between variables was observed (p>0.10) (table 2 and figure 2).

DISCUSSION

All cultures were negative indicating that there were no viable mycobacteria in the samples of milk and milk products selected for this study, in contrast with other researchers who isolated MAP from commercial pasteurized milk and dairy products (Grant *et al*., 2002a; Grant *et al*., 2002b; Ellingson *et al*., 2005;

Variable		IS900-PCR Positive	OR (CI95%)	P value
Time of the year	S-S	3.1% (6/192)	13.41 (0.75 –239.88)	0,07
	A-W (Ref.)	0% (0/192)		
Brand	Second	0.5% (1/192)	0.19 (0.02–1.69)	0,13
	First (Ref.)	2.6% (5/192)		
Thermal treatment	UP	1.56% (3/192)	1 (0.19 – 5.01)	1
	UHT (Ref.)	1.56% (3/192)		
Fat content	Whole	1.56% (3/192)	1 (0.19 – 5.01)	1
	Skim (Ref.)	1.56% (3/192)		

Table 1. Statistical analysis for IS900-PCR.
OR: odds ratio; CI95%: confidence interval 95%; Ref.: reference category for the calculation of the OR.

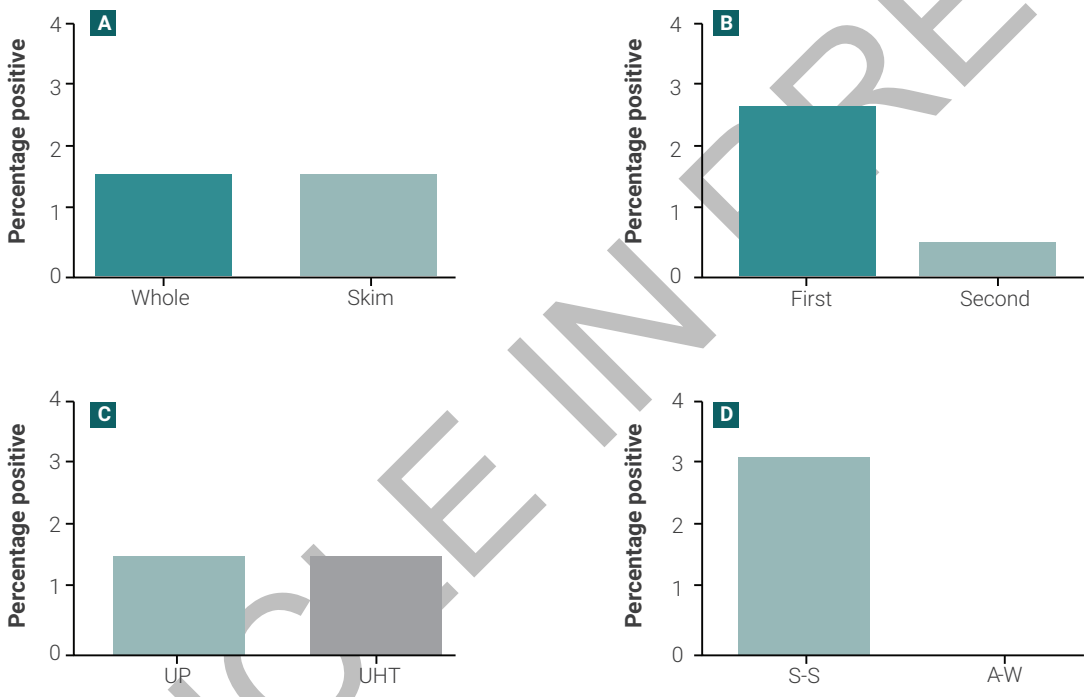

Figure 1. Percentage of IS900 MAP positive samples from retail milk. Results for each group reported as a percentage of the total sample set (n=192). Data were grouped by A. Fat content (Whole, Skim). B. Commercial quality brand (First or high quality, Second or low quality). C. Thermal treatment (UP: Ultra pasteurized, UHT: Ultra High Temperature). D. Time of the year (S-S: Spring-Summer, A-W: Autumn-Winter).

Variable		AAC Lim Positive	OR (CI95%)	P value
Time of the year	S-S	10.9% (21/192)	7.73 (2.26 –26.39)	<0.01
	A-W (Ref.)	1.6% (3/192)		
Brand	Second	12.0% (23/192)	25.9 (3.47–194.55)	<0.01
	First (Ref.)	0.5% (1/192)		
Thermal treatment	UP	8.3% (16/192)	2.09 (0.87 – 5)	0,08
	UHT (Ref.)	4.2% (8/192)		
Fat content	Whole	5.7% (11/192)	0.83 (0.36 to 1.91)	0,67
	Skim (Ref.)	6.8% (13/192)		

Table 2. Statistical analysis for MTBA counts.
OR: odds ratio; CI95%: confidence interval 95%; Ref.: reference category for the calculation of the OR.
Time of the year (S-S: Spring-Summer, A-W Autumn – Winter).

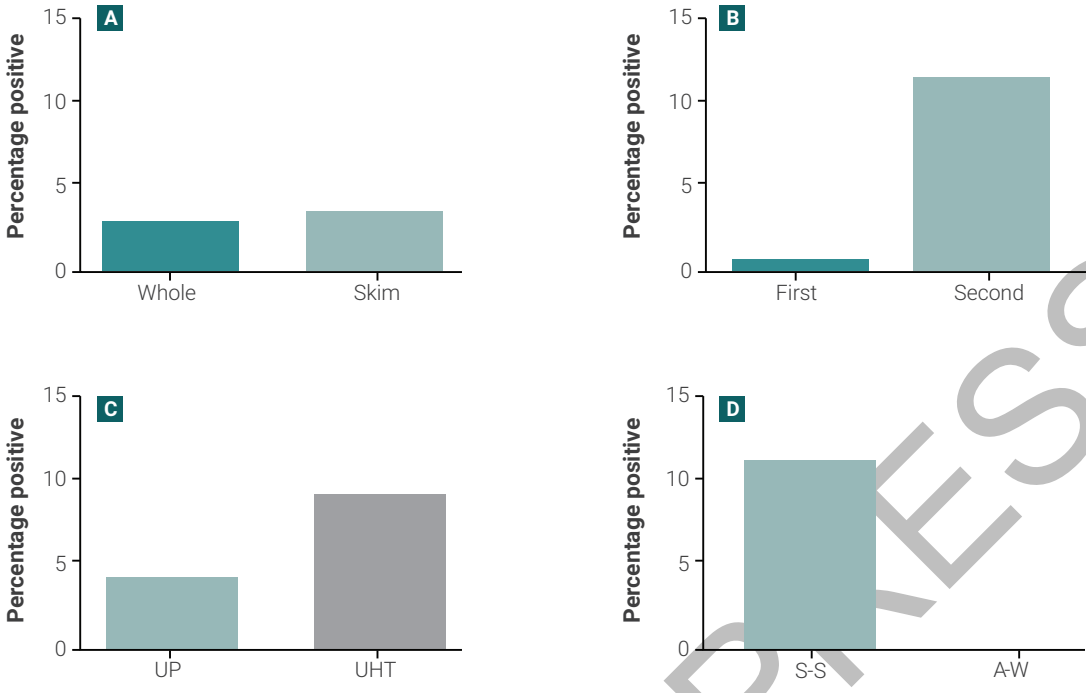

Figure 2. Percentage of samples "Not apt for consumption" by MTBA count from retail milk. Results for each group reported as a percentage of the total sample set (n=192). Data were grouped by A. Fat content (Whole, Skim). B. Commercial quality brand (First or high quality, Second or low quality). C. Thermal treatment (UP: Ultra pasteurized, UHT: Ultra High Temperature). D. Time of the year (S-S: Spring-Summer, A-W: Autumn-Winter). *p<0.01.

Ayele *et al*., 2005; Shankar *et al*., 2010; Hruska *et al*., 2005, Carvalho *et al*., 2012; Paolicchi *et al*., 2012; Serraino *et al*., 2017; Gerrad *et al*., 2018). The surveys for MAP identification in retail milk show that pasteurization is not efficient to inactivate all MAP cells present in raw milk. The samples with a load of mycobacteria under the detection limit would result negatively. Besides, MAP is forming clumps is not uniformly distributed in different matrices like milk products (Grant *et al*., 2002c). Other factors that are affecting the MAP isolation could be genetic differences between MAP strains, effect of decontaminating agents, and interference of other microorganisms that compete with MAP during the incubation (Dundee *et al*., 2001; O´Reilly *et al*., 2004, Carvalho *et al*., 2012). All these factors could contribute to obtaining false negative cultures of mycobacteria. Given that low numbers of MAP are likely to survive pasteurization, testing as large a volume of milk as possible should increase the chances of detecting surviving MAP (Serraino *et al*., 2017). Besides, in comparison, other studies in which MAP was successfully isolated from retail milk have used high quality nutrient and more sensitive culture media (Grant *et al*., 2002; Grant *et al*., 2002b; Ellingson *et al*., 2005; Ayele *et al*., 2005).

The IS900-PCR for MAP detection was positive in 1.56% (6/384) of the milk samples, while all the samples of commercial yogurt and cheeses were negative. No significant effect of the variables was observed (p>0.10). MAP has been detected by PCR between 3% and 15.5% (Anzabi and Hanifian, 2012; Carvalho *et al*., 2009; Gao *et al*., 2002; Grant *et al*., 2002; Millar *et al*., 1996). In Iran, 10.7% of commercial milk samples were positive to PCR (Anzabi and Hanifian, 2012) and In the United Kingdom, 7.05% (Millar *et al*., 1996) but not by culture. The MAP-positive samples would also include samples where DNA from inactivated cells were detected, explaining the high prevalence reported in these studies (Gerrard *et al*., 2018).

In the present study, MAP and mycobacteria were neither detected in samples of commercial cheeses nor yogurts, suggesting that pasteurized milk or dairy products are not a significant source of mycobacteria for consumers. In similar studies carried out in commercial cheeses, MAP was detected between 4% and 50% of the samples by PCR (Ikonomopoulos *et al*., 2005; Farias *et al*., 2014) and between 3-4% by culture (Ikonomopoulos *et al*., 2005; Farias *et al*., 2014). On the other hand, the MAP search was only carried out by Shankar *et al*. (2010), who isolated MAP from 5/9 (56%) milk samples.

Regarding TAB counts, we found that 6.25% of the milk samples exceeded the limits established by the AAC (AAC, 1992). These positive commercialized milk samples could be inefficiently processed or improperly stored, being not suitable for human consumption. A significant effect of the season was observed, being more likely to find samples that exceed the AAC limits from Spring-Summer than Winter-Autumn. There was also a significant effect of the brand on the TAB count, finding that second brand or lower quality products had significantly higher TAB counts than the first brand milk (OR=25.9) (figure 2 B). These results agreed with Freitas *et al*. (2009), who evaluated TAB counts in milks marketed in Brazil, finding that lower quality brands presented higher counts. The genus of bacteria identified in dairy products are environmental microorganisms indicating that the contamination of the samples could be originated during the elaboration and storage of the products.

CONCLUSIONS

All samples were negative for mycobacteria culture, which could indicate that there were not viable mycobacteria or that their concentration was low in the dairy product studied. The PCR for MAP identification was positive for 1.56% of commercial milk samples indicating that the milk came from dairy farms with JD. The absence or low levels of mycobacteria in pasteurized milk, suggests that pasteurized milk or dairy products are not a significant source of mycobacteria to consumers. We found milk samples that exceed the limits established by the AAC for TAB counts. A large number of samples should be analyzed to confirm the correct processing of retail milk. Emphasis should be placed on the sanitary control of diseases caused by bacteria in dairy herds as well as in dairies, with the aim of protecting consumers against the possible risk of infection with potentially pathogenic bacteria.

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