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Molecular detection of *Listeria monocytogenes* in refrigerated raw milk and geospatial distribution in Brazilian small farms

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Abstract

Listeriosis is a zoonosis whose etiological agent is the bacterium *Listeria monocytogenes*. Milk and dairy products are considered excellent substrates for the development of *L. monocytogenes*. Using conventional PCR, *L. monocytogenes* was investigated in 102 samples of raw milk from bulk tanks of small farms. Also, microbiological analysis was performed in positive samples by PCR. The primers such as PRS, LM1/LM2, inlA, inlC, and inlJ were used for spatial analysis. PRS indicated a positivity of 20.58%, and inlJ and inlC primers indicated 21.57%. The primers inlA and LM1/LM2 were negative. Our findings indicate it was possible to achieve effective molecular detection of *L monocytogenes* in the tank milk samples evaluated, in detriment to microbiological culture, in which there was no isolation.

Keywords: listeriosis; bulk tank milk; bovine; molecular; polymerase chain reaction; DNA.

Practical Application: Bulk tank milk may represent a source of contamination of *Listeria monocytogenes*.

1 INTRODUCTION

Listeria species (spp.) are short, rod-shaped, gram-positive bacteria, facultatively anaerobic, non-spore forming, and are generally motile because of peritrichous flagella at a temperature range of 24–28°C but non-motile above 30°C. They belong to the phylum *Firmicutes*, class *Bacilli*, and order *Bacillales* (Matle et al., 2020).

The genus *Listeria* consists of 20 species, which include *Listeria monocytogenes*, *L. marthii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. costaricensis*, *L. ivanovii*, *L. grayi*, *L. rocourtiae*, *L. fleischmannii*, *L. newyorkensis*, *L. weihenstephanensis*, *L. floridensis*, *L. aquatica*, *L. thailandensis*, *L. cornellensis*, *L. riparia*, *L. booriae*, *L. goaensis,* and *L. grandensis*. Of the *Listeria* species identified so far, only *L. monocytogenes* can cause infection in both humans and animals (Matle et al., 2020).

Listeria spp. are the causative agents of the disease called listeriosis. They can be found mainly in soil, but have also been found in water, sewage, and decaying vegetation. They have also been identified in a variety of animals, including ruminants, birds, marine life, insects, ticks, and crustaceans. Because of this, it is considered a zoonotic disease that can be acquired mainly through the consumption of food contaminated with *L. monocytogenes.* Other possible routes of human contamination include direct contact with infected animals and environments and by congenital or transplacental form (Matle et al., 2020; Wang et al., 2021).

L. monocytogenes are potentially pathogenic to humans and animals, and as such have high importance to public health. They can infect various hosts, causing enteric and neurological problems, including diarrhea, septicemia, and miscarriage, with high mortality rates (Bagatella et al., 2021). Its public health significance is due to the high rates of hospitalization and deaths caused by infection, which create significant barriers to socioeconomic development worldwide, requiring stringent regulations involving microbiological standards or criteria for contamination of food products (Matle et al., 2020). Among these species, only those with hemolytic capability are pathogenic (Maćkiw et al., 2021; Oliveira et al., 2020).

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The consumption of raw milk and its derivatives processed without heat treatment or with inadequate control measures is associated with outbreaks of human listeriosis. It is estimated that 9.51 billion liters of uninspected milk were sold in Brazil in 2019 (EMBRAPA, 2020), a situation that poses risks to consumers because of the lack of assurance of proper hygienic conditions.

Raw milk can be contaminated during milking, during storage in bulk tanks, by residual water from milking equipment and utensils, and poorly disinfected teats. In dairy industries, the main routes of infection by these pathogens are contaminated raw milk, utensils, and equipment; soil clinging to the clothes and footwear of workers or visitors; ventilation systems; pooled water or dew; and transport vehicles. Some strains of *L. monocytogenes* can colonize and remain in processing areas for months or even years, making it a significant challenge to eradicate these bacteria (Ruusunen et al., 2013).

The aim of this study was to investigate the presence of *L. monocytogenes* in raw bovine milk from storage tanks of small dairy farms.

2 MATERIALS AND METHODS

2.1 Ethical approval

This study was approved by the Ethics Committee (CEUA) of the Botucatu School of Veterinary Medicine and Animal Science of the Universidade Estadual Paulista "Júlio de Mesquita Filho" (FMVZ/UNESP), under registration number 18/2021.

2.2 Samples of cow's milk from bulk tanks

Milk samples were obtained from 102 individual bulk tanks, with one sample per farm, located in eight municipalities in the midwest region of the state of São Paulo, Brazil: Avaré (n = 47), Cerqueira Cesar (n = 10), Arandu (n = 4), Bofete (n = 7), Pardinho (n = 16), Anhembi (n = 13), Botucatu (n = 2), and Bauru (n = 3) (Figure 1).

The milk samples were collected directly from individual bulk tanks with a maximum temperature of 4°C, containing milk from cows milked in the previous 48 h. Before collecting, the tank agitator was activated to homogenize the milk for at least 5 min, after which a sterilized stainless steel ladle was used to collect 250 mL of raw milk, which was placed in a sterile plastic jar. The jars were then placed in an isothermal chest containing recyclable ice packs and taken to the laboratory for the analytic procedures.

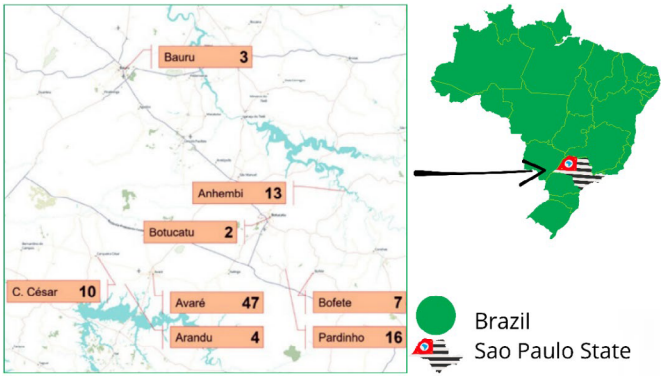

Figure 1. The samples were collected in eight municipalities located in the state of São Paulo, Brazil.

2.3 Molecular tests

2.3.1 Extraction of DNA from the milk to identify L. monocytogenes

The DNA from the milk samples was extracted with the commercial kit Illustra Blood GenomicPrep (GE Healthcare®), according to the protocol indicated by the manufacturer, with slight modifications as suggested by Cunha et al. (2006).

2.3.2 Quantification of DNA

The quantity and concentration of nucleic acid extracted from each sample were determined by spectrophotometry, using a NanoVue Plus™ device (GE Healthcare Biosciences, UK), according to values obtained from the A260/280 ratio, according to Desjardins and Conklin (2010). All the DNA samples were kept frozen at -20°C until used for the PCR analysis.

2.3.3 Selection of primers

The primers were chosen based on specificity and were submitted to the GenBank via the BLAST® and Primer-BLAST® options of the National Center for Biotechnology Information (NCBI).

The following oligonucleotide primers were employed:

- PR(f/r), selected with the objective of recognizing the species by studying the gene *prs* (phosphoribosyl pyrophosphate synthetase), common to the genus *Listeria* spp. according to Doumith et al. (2004);
- LM1/LM2 (Border et al., 1990), selected with the goal of studying the gene *hlyA* (*Imo0202*), which encodes listeriolysin O, one of the main virulence factors related to the adhesion, invasion, escape of primary vacuoles, and suppression of the host's immune response (Liu et al., 2017; Wu, 2015);
- inlA, inlC, and inlJ, genes that encode internalins, responsible for the internalization, with the objective of determining the virulence of the strain, according to Liu et al. (2007).

2.3.4 Controls

As positive controls, we used *L. monocytogenes* strain ATCC 7644, provided by the Laboratory of Food Inspection (SOAP) of FMVZ—UNESP—Botucatu, and DNA extracted from *L. monocytogenes* strain ATCC 19117, provided by the Laboratory for Inspection of Products of Animal Origin (UEL)—Londrina—Paraná. Ultrapure sterilized water was used as a negative control.

2.3.5 Conventional PCR

For the PCRs of the oligonucleotides *PRS(f/r)* and LM1/LM2, we used an individual 0.2 mL reaction tube for each

sample, which received PCR buffer (50 mM of KCl, 20 mM of Tris-HCl), 1.6 mM of $MgCl_2$, 0.2 mM of dNTPs, 1 µL of Taq-polymerase (Platinum Taq DNA Polymerase, Invitrogen®), 0.2 µM of each primer (forward and reverse), 1 µL of sample, and 8.3 µL of ultrapure water (MIX-PCR). Each tube thus contained 12 µL (11 µL of MIX-PCR and 1 µL of the product extracted from the DNA of the sample tested).

For the PCRs of the oligonucleotides *inlA*, *inlC,* and *inlJ*, we used an individual reaction tube for each sample, which received PCR buffer (50 mM of KCl, 20 mM of Tris-HCl), 0.75 mM of $MgCl_2$, 0.5 mM of dNTPs, 0.5 µL of Taq-polymerase (Platinum® Taq DNA Polymerase, Invitrogen®), 0.5 µM of each primer (forward and reverse), 2 µL of the sample in question, and 17.5 µL of ultrapure water (MIX-PCR). Therefore, each tube contained 25 µL (23 µL of MIX-PCR and 2 µL of the product from the extraction of the DNA from the sample tested).

The reactions were performed with a Mastercycler Pro Gradient thermocycler (Eppendorf®). For the primers *PRS(f/r), inlC,* and *inlJ*, the reactions were based on the protocol described by Doumith et al. (2004); for the primer *LM1/LM2*, the protocol was based on Aznar and Alarcón (2003); and for the primer inlA, it was based on Liu et al. (2007).

2.3.6 Agarose gel electrophoresis

Identification of the products amplified by PCR was performed using 2% agarose gel electrophoresis. Products from the amplified *L. monocytogenes* strains were used as positive controls and sterile milli-Q water as a negative control. The size of the amplified fragments was verified by visual comparison with molecular weight standards (ladders) and with the *L. monocytogenes* strains used as positive controls.

2.3.7 Sequencing

The cPCR products were purified with the enzyme ExoSap (USB), utilizing 4 µL of the enzyme for each 10 µL of cPCR product, by initial incubation for 1 h at 37°C followed by 20 min at 80°C, and then quantified by spectrophotometry. The samples selected were the cPCR products that were most strongly amplified.

The amplicon images were visualized using the Chromas 2.3 software in the form of an electropherogram aligned by the MEGA program (Molecular Evolutionary Genetics Analysis), version 10.0 (Kumar et al., 2018). They were subsequently submitted to BLASTn (http://www.ncbi.nlm.nih.gov/BLAST) and compared with the sequences deposited in the relevant databases.

2.3.8 Microbiological analysis

Samples that were positive according to more than one primer were submitted to microbiological analysis through culturing for isolation of *L. monocytogenes*, with the purpose of evaluating the phenotypic characteristics of the colonies and viability of the bacteria, according to the MFHBP-30 method, as described by Pagotto et al. (2001). Briefly, 90 mL of *Listeria* enrichment broth (LEB) was added to 10 mL of each sample and incubated at 30°C for 48 h. After this period, 0.1 mL of cultured LEB was transferred to a flask containing 10 mL of Fraser *Listeria* selective enrichment broth and incubated at 35°C for 48 h. In this step, it was already possible to confirm the presence or not of the genus, and when there was suspicion of a positive sample (alteration of color of the Fraser medium), it was submitted to seeding in dishes containing Palcam agar and Oxford agar, followed by incubation at 35°C for 24-48 h.

2.3.9 Spatial analysis

The data were submitted to geoprocessing through the Geographic Information System (GIS) with the QGis 3.16.1 software for mapping the positive samples to understand the spatial distribution of the positive cases in the study area.

2.3.10 Statistical analysis

The data were transformed into descriptive statistics by calculating the frequencies and percentages of each primer and municipality through the SAS® OnDemand for Academics software.

The statistical analysis showed that 50 milk samples (49%) were positive based on at least one primer, i.e., nearly half of the milk samples contained the bacterium. The most frequent primer was inlJ, with 23 positive samples (22.55%), followed by inlC with 22 positive samples (21.57%), and PRS with 21 positive samples (20.59%).

Of the municipalities surveyed, the greatest prevalence was found in Anhembi, where 76.92% of the samples were amplified by at least one primer, followed by Avaré with 51% positive samples, Cerqueira Cesar and Botucatu with 50%, Bofete with 42.85%, Pardinho with 37.50%, and Arandu with 25%. None of the samples collected in Bauru were positive. In total, 12 milk samples were amplified by more than one primer, of which six (50%) came from Anhembi, four (33%) were from Avaré, and one each was from Cerqueira Cesar and Pardinho. Two samples were amplified by three primers (PRS, inlC, and inlJ), both from Pardinho.

3 RESULTS

There was no isolation of *L. monocytogenes* in the microbiological analyses. Of the 102 samples tested, there were 21 milk samples positive for the primer PRS, which amplifies a segment of the gene *prs* (*Imo0509*), common to the genus *Listeria* spp., resulting in a prevalence of 20.59%, while 23 samples were positive for the primer inlJ, which amplifies a segment of the gene *Imo2821*, resulting in a prevalence of 22.55%, and 22 samples were positive for the primer inlC (*Imo1786*), with a prevalence of 21.57%.

The samples that were positive for the gene *inlJ* were 100% similar to *L. monocytogenes* (access key: CP054846.1). For the primer inlC, the sample selected was 91, which showed 100% similarity (access key: CP0054040.1). The spatial analysis of the municipalities of the dairy farms evaluated allowed detecting *L. monocytogenes* in tank milk samples, confirming positivity in all the municipalities except Bauru, namely Avaré, Cerqueira Cesar, Arandu, Bofete, Pardinho,

Anhembi, and Botucatu. Figure 2 depicts the municipalities of the farms with tank milk samples positive for *L. monocytogenes* according to PCR.

4 DISCUSSION

The results showed the presence of *L. monocytogenes* DNA and some of the genes important for its manifestation as a pathogen in tank milk intended for human consumption.

Our bibliographic review revealed that the presence of the pathogen in raw milk has been detected with highly variable prevalence in countries for which data are available. In the United States, studies of raw milk in bulk tanks have reported the presence of *L. monocytogenes* ranging from 0 to 19.7% (Lee et al., 2019).

In Brazil, the literature review conducted by Barancelli et al. (2011) reported occurrence from 0 to 37% in raw milk and from 0 to 41% in cheese samples. More recent studies have not found any positive milk samples (Oliveira et al., 2020) or cheese samples (Oxaran et al., 2017).

Various environmental sources have been reported as possible reservoirs and origins of contamination by *L. monocytogenes* during milking and storage of milk (Bagatella et al., 2021; Castro et al., 2018; Rodríguez et al., 2021; Whitman et al., 2020). Traits such as the ability to grow in a wide temperature range, intracellular habit, facultative anaerobia, and halotolerance give the pathogen strong resistance and great adaptability to hostile environments, allowing its widespread diffusion, especially in dairy farms (Colagiorgi et al., 2016; Radoshevich & Cossart, 2018; Rodríguez et al., 2021).

Water and soil are places where the bacterium is commonly isolated, directly exposing livestock through their feeding in pastures. Silage with poor quality is also considered an important source of infection. The infected animals eliminate the pathogen in their feces, potentially contaminating soil, crops, and water sources, perpetuating the cycle (Castro et al., 2018; Rodríguez et al., 2021).

The presence of *L. monocytogenes* in the udders of animals before milking has been described as the main risk factor for its presence in milk. The bacteria, through cross-contamination, can be present due to poor hygiene before milking or via intramammary infection (Castro, 2022).

Contaminated silage has repeatedly been identified as a source of animal infection. When ingested, the pathogen reaches the udder via the bloodstream, with consequent excretion in the milk (Bagatella et al., 2021).

Contaminated raw milk, when intended for the production of dairy products, can be a source of contamination of milking equipment, filters, bulk tanks (Rodríguez et al., 2021; Whitman

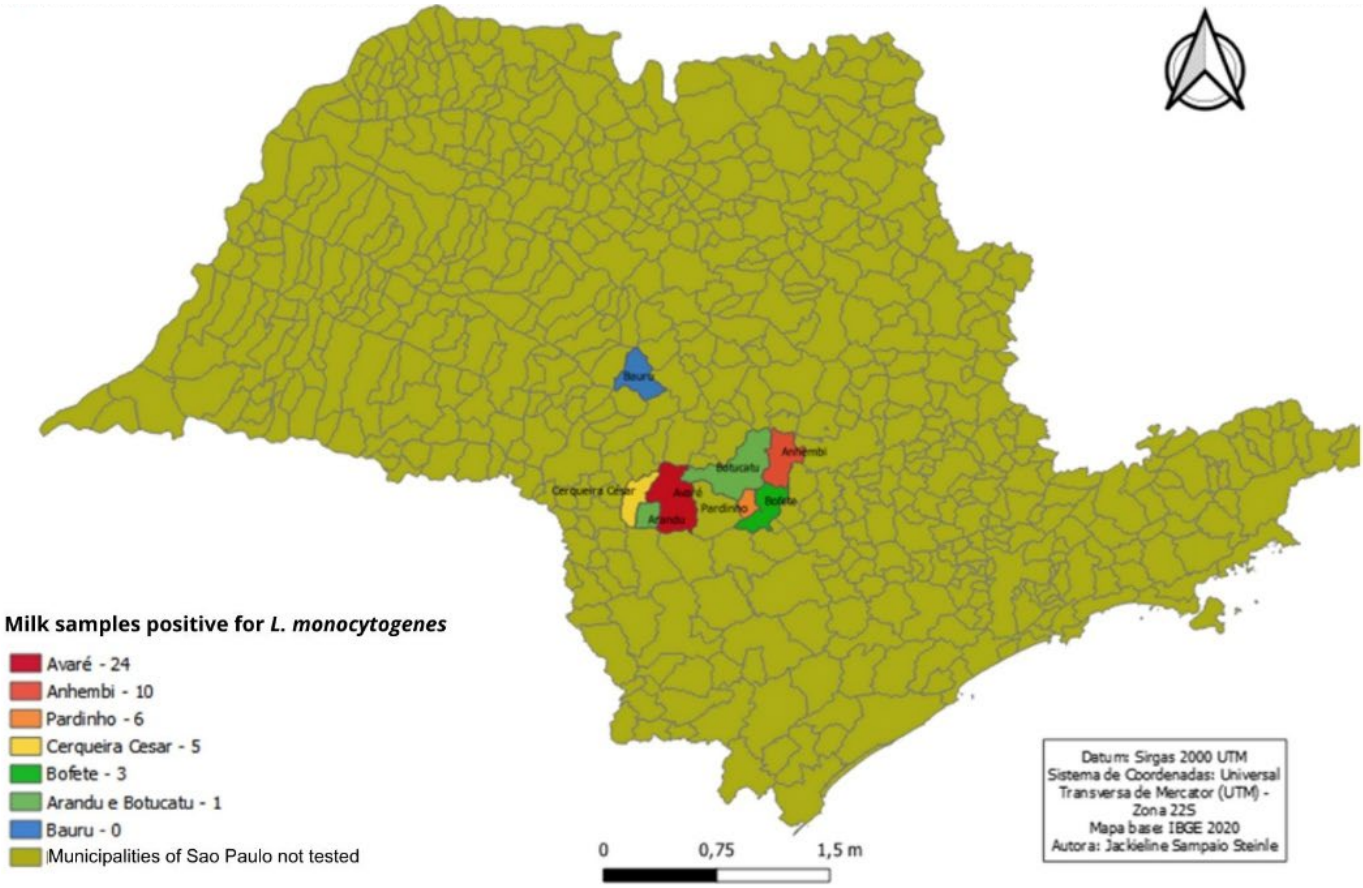

Figure 2. Number of tank milk samples positive for *L. monocytogenes* and the respective municipalities of origin.

et al., 2020), and plants producing dairy products. This contamination can be aggravated by the ability to form biofilms by strains of *L. monocytogenes*, enabling greater adhesion to surfaces and resistance to sanitizers (Castro et al., 2018).

There has been a good deal of discussion regarding standardizing a fast and efficient protocol to detect genes indicative of the virulence factors of *L. monocytogenes* in foods and the environment. However, the wide variety of factors that affect the pathogen's virulence and the sensitivity of PCR make this a challenge, as observed in several studies (Chen et al., 2017; Dhama et al., 2015).

It is important to mention that when we followed the PCR cycling protocol described by Liu et al. (2007), for the primers inlC and inlJ, there was no amplification of the positive control, so we employed the cycling described by Doumith et al. (2004) and managed to obtain amplification of the target DNA of the amplicons. It should be considered that various factors can influence the results of molecular analyses, such as the primer choice or quality of the sample. Inhibitors have also been reported negatively on the sensitivity of the PCR technique (Aznar & Alarcón, 2003).

The milk samples indicated as positive in the molecular tests for more than one primer were submitted to microbiological culturing for *L. monocytogenes* with the purpose of evaluating the phenotypic characteristics of the colonies and the viability of the bacterium. However, it was not possible to isolate *L. monocytogenes* based on the samples from the molecular tests that were positive for more than one primer. This can be considered a limitation of the study, considering the microbial competition due to the large quantity of bacteria present in the tank milk, even though we used media specific for the isolation of *Listeria,* as well as the sensitivity of bacteria of the genus *Listeria* to competition with the contaminating microbiota and natural microbiota of raw milk (Allison et al., 2018). Besides this, the bacterial viability might have been influenced by the interaction of factors, such as pH, water activity, osmolarity, number of microorganisms, and storage conditions, that affect their survival (Ekonomou et al., 2020). Freezing can also submit cells to stress factors and cause injuries, such as mechanical shear, osmotic alteration, and damage to the cell walls or membranes caused by mechanical stress of the ice crystals formed outside or within the cells, all of which can cause their inactivation (Boziaris et al., 2021).

Although the sale of raw milk and its derivatives is restricted in Brazil, they still can be purchased clandestinely, including on the internet and social media platforms, such as Facebook Marketplace. This type of trade is widespread in the country, mainly in the Southeast region where this study was conducted (Fagnani et al., 2022). This situation raises questions about the health risks associated with raw and dairy products. Accordingly, the high frequency of sales together with the detection of nucleic acid of *L. monocytogenes* must be interpreted as an alert, highlighting the importance of periodic monitoring surveys.

In relation to good manufacturing practices at establishments producing dairy products, the area of receiving the raw milk and other inputs should be effectively separated from the final production area, to prevent cross-contamination resulting from counterflows, associated with a program to monitor both the environment and final products (Leong et al., 2017; Maćkiw et al., 2021).

Although refrigeration can control the growth of various pathogenic agents, some can survive and multiply satisfactorily at cold temperatures, as is the case with *L. monocytogenes*, which can act as a contaminant at various steps of obtaining and processing milk. The presence of these bacteria suggests the existence of poor hygiene (Reguillo et al., 2018; Rodríguez et al., 2021).

Rigorous measures for environmental hygiene, health of herds, implementation of good dairy manufacturing practices, tracing of bacteria in the environment and animals, and heat treatment of milk are crucial to prevent dissemination of pathogens and control health risks of consuming raw milk and its use to make dairy products (Rodríguez et al., 2021).

To achieve these goals, educational measures and hygiene-sanitary programs need to be implemented among small farmers (Brasil, 2017). The success of such measures can be judged by molecular detection of the pathogen, to evaluate the potential risks to human health and guide the implementation of prevention and control measures for storage and processing of raw milk, to minimize contamination by *L. monocytogenes.*

Analysis of outbreaks of listeriosis in the world has demonstrated the possibility of foodborne dissemination of the disease (European Food Safety Authority & European Centre for Disease Prevention and Control, 2019; WHO, 2018). Although there are no data correlating listeriosis with the consumption of contaminated foods in Brazil, the presence of *L. monocytogenes* has often been reported in the country, leading us to believe the disease is underreported (Oliveira et al., 2020; Oliveira & Silva, 2022). Nevertheless, the relevant national legislation does not include comprehensive programs to monitor this pathogen in animal products, instead only specifying criteria for acceptance of some types of cheese (Brasil, 2001). Hence, there is a need for stronger monitoring and control measures.

Tools that enable tracking of *L. monocytogenes* strains and determining their origin by testing potentially contaminated samples can serve to guide centralized actions targeted at farms with greater occurrence of the pathogen.

Regarding the spatial analysis, tank milk samples from all the municipalities except Bauru were found to be positive by molecular detection of *L. monocytogenes*. However, this was likely due to the study design, since we only collected three samples from this municipality.

Although our results improved on previous publications, some limitations need to be considered. Initially, DNA-based tests can identify both living and non-living pathogens, which can be beneficial considering the cultivable characteristics of *L. monocytogenes*, considered to be time-consuming and labor-intensive. However, this can also lead to some disadvantages. Detecting genetic material from non-viable cells can also result in positive test results. But it does not detract from the fact that there is circulation of *L. monocytogenes* strains in raw milk. Thus, more

research is needed to better understand the sanitary risks of dairy manufacturing regarding the cross-contamination of raw milk.

In addition, it is also important to consider the geographic limitations of a survey conducted using a convenience sample, which can limit the applicability of the findings to other settings or populations. However, considering the diversity of municipalities together with the considerable number of samples (102) and its geographical distribution, we believe that the conclusions drawn from our research suggest a scenario that is reasonably close to the reality of the midwest region of the state of São Paulo.

5 CONCLUSION

We were able to achieve effective molecular detection of *L. monocytogenes* in the tank milk samples evaluated, in detriment to microbiological culture, by which there was no isolation. The molecular detection of this pathogen thus indicates a need to implement educational measures to support efforts to minimize contamination by *L. monocytogenes* from the storage and processing of raw milk.

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