

## Occurrence of *Listeria monocytogenes* in cheese and ice cream produced in the State of Paraná, Brazil

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*The occurrence of Listeria monocytogenes in Brazilian ice cream and in some soft and semi-soft cheeses produced and sold in the State of Paraná, Brazil was evaluated. Ninety samples of cheese and sixty samples of ice creams were analyzed following the guidelines outlined by the official institutes, AOAC and FDA. In the ice cream samples no isolation of Listeria spp. was found. The percentage of these ninety samples of cheeses positive for Listeria spp. was 12.20%. Listeria monocytogenes was detected in six (6.70%) of the same samples. The presence of Listeria innocua was five (5.50%) in the samples analyzed was also observed. According to the results of the work it is possible to presume that there is a potential health risk to the Brazilian population, heightened by aging and the increase in immunodepressed. These results indicate the need for the implementation of monitoring of these microorganisms as much by producers as by health inspectors. The results also show that the VIP (visual immunoprecipitation assay) is a viable triage method of contaminated samples for the liberation of products for commercialization, as it is quick, reliable and does not require additional equipment other than that normally found in production labs, while presenting reliable results.*

### Uniterms

- *Listeria monocytogenes*/ occurrence
- Cheese/microbiological analysis
- Ice cream/microbiological analysis

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## INTRODUCTION

Microorganisms of the genus *Listeria* are widely distributed in nature, although with a limited number of species of medical and veterinary significance (Schuchat *et al.*, 1991; Low, Donachie, 1997; McLaughlin, 1997).

Many studies cite food products, especially cheeses

and other milk based products as the principal sources of *Listeria monocytogenes* transmission (Ho *et al.*, 1986; Azadian *et al.*, 1989; Ryser, Marth, 1990).

The incidence of *Listeria monocytogenes* in soft and semi-soft cheese varied from 0.50% to 46.00% (Terplan *et al.*, 1986; Farber *et al.*, 1987; Beckers *et al.*, 1987; Weber *et al.*, 1988; Pini, Gilbert, 1988; Rorvik, Yndestad, 1991;

Cordano, Rocourt, 2001; Guerra *et al.*, 2001; Manfreda *et al.*, 2005; Pintado *et al.*, 2005; Colak *et al.*, 2007). Studies related to the occurrence of *Listeria monocytogenes* in cheeses produced in Brazil have shown contamination of 1.40% to 41.17%. (Destro, 1991; Oliveira, 1993; Schwab, 1994; Silva *et al.*, 1998; Souza, 2002; Borges *et al.*, 2003; Branco *et al.*, 2003; Ramos, Costa 2003).

Data provided by the Brazilian Association of the Cheese Industry – ABIQ, indicate that Brazilian production in 2005 was around 510,000 tons of cheese. The types of cheese with the highest consumption include mozzarella, table, Brazilian Minas, soft cream cheese and cream cheese for culinary purposes. The production of cheese is concentrated, mainly, in small and medium sized industries, in the South and Southeastern regions of Brazil.

Resolution number 12 of January 2<sup>nd</sup>, 2001 (Brasil, 2001) establishes the microbiological standards for foods intended for human consumption and defines the search for *Listeria monocytogenes* (absence or presence in 25 g) only in the cheese sub-group, specifically those which present medium, high and very high humidity.

Ice cream, despite its frozen storage, is also a very good medium for microbial development, thanks to its high nutritional level, its nearly neutral pH (6-7) and to its long period of storage. In 2001, the Brazilian National Health Vigilance Agency - ANVISA monitored 863 samples of frozen food, and detected 23.00% which were in disagreement with the microbiological standards (Brasil, 2002).

Windrantz and Arias (2000) performed a bacteriological evaluation of ice cream from San Jose, Costa Rica, and obtained a 12.30% occurrence of *Listeria monocytogenes*. All the isolates were obtained from home-made ice cream samples.

Despite the risk of *L. monocytogenes* in dairy products, there are few studies about its incidence in cheese and ice cream produced in Paraná, Brazil.

The aim of this work was to determine the occurrence of *Listeria monocytogenes* in different kinds of cheese and ice cream produced in the State of Paraná, Brazil using the quick VIP (visual immunoprecipitation assay) Biocontrol in the sample triage.

## MATERIAL AND METHODS

During 2001 and 2002, ninety samples from different kinds of cheese were collected in establishments selling food in a number of 16 regions of the State of Parana (Apucarana, Cascavel, Cianorte, Cornelio Procopio, Curitiba, Foz do Iguaçu, Francisco Beltrão, Guarapuava, Irati, Ivaipora, Londrina, Maringa, Paranavai, Pato Branco, Toledo and Umuarama); and in 2004, sixty

samples of ice cream were collected in small companies of frozen foods in the Curitiba area. All samples of collected cheeses were produced in this State and were classified according to the Brazilian Cheese Industry Association criteria as: mozzarella (19), table (6), cream cheese (1); fresh cheeses subdivided into, Brazilian Minas (25) and ricotta (11); handcrafted cheese, subdivided into: colonial (23), mountain (2), standard Minas (1), gouda (1) and provolone (1). The samples were packed in first use plastic bags, tagged and kept refrigerated during the transportation to the laboratory, and then analyzed immediately. The humidity of the cheese samples was determined in a vacuum stove. The humidity content of the cheeses was classified as follows: low humidity cheese, when the humidity content was lower than 36.00%, medium humidity cheese, when the humidity content was between 36.00 and 46.00%, high humidity cheese, when the humidity content was between 46.00 and 55.00%, and very high humidity cheese when the humidity content was higher than 55.00% (Brasil, 2001).

The detection of *Listeria monocytogenes* and related *Listeria* spp was performed using the rapid visual immunoprecipitation assay (VIP) / AOAC 997.03 method as a screening technique. This method uses a proprietary reagent system to form an antigen-antibody-chromogen complex if *Listeria* is present. Appropriately enriched samples are added to the VIP unit; any *Listeria* antigens will bind to the antibody-chromogen complex as it flows across a supporting membrane. When *Listeria* is present, the antigen-antibody-chromogen complex will form a detection line in the test sample window. Sample flow will continue down the membrane to form a control line in the test verification window regardless of whether the sample contains *Listeria* (Warburton, Boville 2003).

Aliquots of 25 g of products were homogenized with 225 mL of modified Fraser broth with lithium chloride 8M for 30 seconds in a “stomacher” and were incubated at 30°C for 28 hours. After the incubation period, the flask with the primary selective enrichment was slightly agitated. Then 1 mL of the homogenized sample was transferred to a test tube with 9 mL of enrichment broth of *Listeria* buffered (BLED) which was incubated at 30°C for 24 hours (secondary selective enrichment). Then, 1 mL of the BLED broth was transferred to a sterile test tube and heated in a water bath at 100 °C, for 5 minutes. An aliquot of 0.2 mL of BLED broth previously heated and cooled, at room temperature, was transferred for inoculation in the VIP<sup>R</sup> Biocontrol device in the specific area with round shape. After 10 minutes, the readout was performed (BioControl Systems, 1997).

The positive BLED broths in the VIP<sup>R</sup> Biocontrol test, which were not previously heated, were plated on three se-

lective media according to the *Food and Drug Administration* (FDA, 1995): (a) selective phenylethane moxalactam lithium chloride agar for *Listeria* spp. and supplemented with esculin 1 g/L, ferric ammonium citrate 0.5 g/L (modified LPM), (b) selective Oxford Agar for modified *Listeria* spp. (MOX; Oxford Agar base; Merck) supplemented with moxalactam (15 mg/L) (Sigma) and colistine sulfate (10 mg/L), and (c) Agar Palcam (PAL; Merck) supplemented with polymyxin B (10 mg/L), acriflavine (5 mg/L), and ceftazidime (20 mg/L) (FDA, 1995).

The Palcam and Oxford agar plates were incubated at 35 °C for 48 h and the LPM agar at 30 °C for 48 h. In the cases of presumptive colony growth, five typical colonies were selected and then purified in tripticase soy agar supplemented with 0.60% of yeast extract and identification tests at genus level were performed: (a) umbrella-like growth in semi-solid sulfite-indol-motility medium with addition of 0.05% of TTC (triphenyltetrazolium chloride) at 25 °C; (b) catalase production; (c) acid production from glucose, lactose and saccharose in agar triple sugar iron (TSI); (d) methyl red test reaction; (e) Voges-Proskauer test and (f) bile-esculin test. The biochemical assays at species level involved: (a) nitrate reduction; (b) hemolysis production in blood agar with Columbia base supplemented with 5.00% of sheep blood; (c) fermentation of xylose, ramnose, and manitol in basic medium of bromocresol purple; (d) synergistic hemolysis test in the CAMP factor (Christie–Atkins–Munch–Peterson test) in sheep blood agar. A rapid serum agglutination test with somatic homologous antiserum was carried out with the pure cultures previously grown in nutrient agar at 30 °C for a period of 18-24 hours. (FDA, 1995)

The isolates identified as *Listeria* spp., were submitted for complementary biochemical characterization in the Adolfo Lutz Institute (SP). The serological characterization was made in the Oswaldo Cruz Institute.

The negative results in VIP assays were accepted as absence of *Listeria* spp in 25 g of samples.

The presence or absence of *L. monocytogenes* in 25 g of food was the criterion used to classify the analysed food articles as appropriate or inappropriate for consumption.

Results were analyzed with Z test for two proportions using the Microsoft Excel. A  $p \leq 0.05$  was considered statistically significant.

## RESULTS AND DISCUSSION

From a total of 90 samples of different kinds of cheese, 11 (12.20%) were positive for *Listeria* sp, subdivided as *L. monocytogenes* in six samples (6.70%), *L. innocua* in five samples (5.50%). From a total of 60

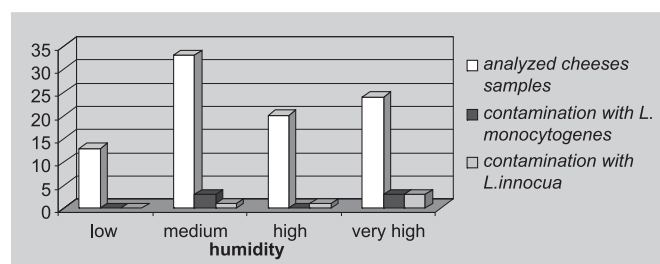
samples of ice cream analyzed, none were positive for *Listeria* spp. The serovar distribution analysis showed that 100% of the *L. innocua* isolates belonged to serotype 6a and for three of the *Listeria monocytogenes* isolates tested, serovar prevalence was 1/2a.

Eleven samples of cheese showed positive evidence of VIP<sup>R</sup> Biocontrol for *Listeria* sp. Positivity was determined through the observation of a gray line in the result hole of the device, indicating the presence of flagellate antigens of *Listeria* spp., as well as the appearance of a gray line in the control hole indicating the correct operation of the device in the readout times of 10 and 20 minutes, respectively.

After selecting five suspect colonies in the selective media for *Listeria* spp. (modified LPM, Oxford agar and Palcam agar) from the 11 BLED broths with positive VIP<sup>R</sup> proof, biochemical identification at genus and species level was performed. In the identification proof, all isolates presented positive results for the genus *Listeria* (catalase, motility in umbrella shape, bile esculin test, production of acid from glucose, lactose and saccharose in TSI agar) differing from the sugar fermentation profile, b-hemolysis and CAMP tests for *S. aureus*. All the strains identified as *L. monocytogenes* produced b-hemolysin and were positive for the CAMP tests, reduction of nitrate to nitrite and fermentation of xylose and manitol in basic medium of bromocresol purple.

The cheeses were classified as low humidity (13), medium humidity (33), high humidity (20) and very high humidity (24). The samples of very high humidity content (26.70%), presented the highest occurrences of *Listeria* spp. (25.00%) and *Listeria monocytogenes* (12.50%). In medium and high humidity cheeses, the *Listeria* spp contamination was 12.12% and 5.00% respectively. *Listeria monocytogenes* was not isolated in high humidity cheeses but in medium humidity samples was 9.09%. *Listeria* spp. was not isolated using this methodology in cheeses with a low humidity content. These results are shown in Figure 1.

Table I and II show the results of statistical analysis looking for any difference determined by the contamination



**FIGURE 1** - Humidity content of cheeses and contamination level with *Listeria monocytogenes* and *Listeria innocua*

degree as function either from the moisture content or from *Listeria* species. No statistical significance ( $p > 0.05$ ) was found.

**TABLE I** - Occurrence of *Listeria* spp. in cheese

Results	<i>Listeria</i> spp.	
	<i>L. monocytogenes</i>	<i>L. innocua</i>
f	6	5
f(%)	54.50	45.40
Z	0.303	
p	0.762	

f- frequency; f(%)frequency (%); Z - Z test for two proportions from one group with mutually exclusive group

**TABLE II** - Occurrence of *Listeria* spp. in cheese as a function of humidity

Results	Humidity	
	Medium	Very high
f	4 (33)	6 (24)
f(%)	12.10	25.00
Z	-1.262	
p	0,207	

f- frequency; f(%)frequency (%); Z - Z test for two proportions from independent groups

Traditional methods for the detection of *L. monocytogenes* in food are based on the use of a specific culture medium for the enrichment and isolation of possible existing microorganisms, followed by biochemical confirmative and serological assays. The greatest disadvantage of the traditional methodologies used to isolate *L. monocytogenes*, is the time necessary to obtain the results. The procedures of enrichment, isolation and biochemical assays, required for the confirmation of the isolated strains, can take two weeks or longer. So, methods are being developed to produce faster and more reliable results and to improve laboratory productivity. Considering that the majority of the products analyzed presented a short "sell by date" period, time spent waiting for the results could compromise product commercialization. Thus, realization of analysis through a faster method was sought. The VIP method was chosen due to the ease in obtaining this reagent system and because it did not require the acquisition of equipment not already available in microbiology laboratories. Screening by the VIP method economizes time, materials and man power. The conventional method will be used only when samples prove positive for *Listeria* spp. VIP

screening method makes detection and identification faster, more convenient, more sensitive, with greater specificity and improves the laboratory productivity. The presence of *L. monocytogenes* in 25 g aliquot classifies these products as inappropriate for consumption in accordance with the Brazilian law (Brasil, 2001) which stipulates the absence of *L. monocytogenes* for cheeses with medium, high and very high humidity contents. Quantification of the pathogen is not required by current Brazilian legislation.

Souza (2002) detected 1.40% of *L. monocytogenes* in 70 samples of hand-made sour cheese sold in Fortaleza – CE, while Ramos and Costa (2003) detected 1.70% in samples of the same kind of cheese sold in Manaus.

Oliveira (1993) detected 2.00% of *Listeria monocytogenes* in Brazilian Minas cheese in the retail business of Goiânia – Goiás. This percentage was also found by Schwab, in 1994, in colonial hand-made cheese samples sold in Porto Alegre. Destro (1991) isolated *L. monocytogenes* in 10.00% of Brazilian Minas cheese samples, while Silva *et al.* (1998) found a higher incidence (41.20%) of *L. monocytogenes* in hand-made Brazilian Minas cheese. However, other authors like Casarotti *et al.* (1994) and Feitosa *et al.* (2003) did not detect this microorganism in samples of Brazilian Minas cheese, sold in Piracicaba, São Paulo, nor in sour cheese and butter produced in the State of Rio Grande do Norte, respectively. Silva *et al.* (2003) collected 218 samples of cheese along the production line and the environment in two factories of Minas Frescal cheese. Thirteen isolates of *Listeria* spp. were found in these samples, of which 70.00% were positive for *L. innocua* and 15.00% for *L. monocytogenes*.

When evaluating the existing research in relation to the occurrence of *Listeria monocytogenes*, a comparative study between them is difficult due to the range of analytical methods used, as well as differences in culture medium, sampling patterns adopted in each case and in the results achieved, which may have resulted in different detection levels. Further, according to Wong *et al.* (1990), differences in occurrence data can also be explained by the geographic specificity of the distribution of the genus *Listeria*.

In relation to the Brazilian studies, these difficulties are accentuated by the existence of typical Brazilian products, like Minas cheese, sour cheese and butter, among others. However, what can be observed in the literature is the predominance of a low incidence of this microorganism, an agreement found in the majority of the results obtained so far.

Despite the ubiquitous nature of the *Listeria* genus, the presence of *Listeria monocytogenes* in cheese is a public health concern. *L. monocytogenes* is a pathogenic bacterium in humans. It can cause listeriosis, which is characterized by fever, muscle ache and gastrointestinal symptoms, such as

nausea or diarrhea. The infection can affect the nervous system, causing headache, confusion, loss of balance or even convulsion. Healthy people are occasionally infected with *L. monocytogenes*, but rarely present serious symptoms. Risk groups consist of elderly people and immunocompromised (transplant patients and those with lymphoma and AIDS). In addition, the contamination in pregnant women, which presents only mild symptoms (like a common cold), can result in the infection of the embryo, leading to spontaneous abortion, premature birth, severe infections of the new-born or stillbirth (Bille *et al.*, 1999). Even though *L. innocua* is not pathogenic to humans, its growth in cheese samples suggests the possibility of the growth of *L. monocytogenes* in these products, representing a potential risk to consumers.

The importance of this work from the public health view point is that it shows that a potential risk to the health of the general population, heightened by the increase in the number of elderly and the immunodepressed for whom *L. monocytogenes* is potentially serious and also by the fact that *L. monocytogenes* isolates which belong to serotype 1/2a were found among those extracted. Furthermore, this is one of the three serotypes most commonly responsible for outbreaks of listeriosis (1/2a, 1/2 b, the most frequent being 4 b) (Kerouanton *et al.*, 1998; Margolles *et al.*, 1998; Hofer *et al.*, 2000).

The results of isolate serotyping (1/2a for *L. monocytogenes* and 6a for *L. innocua*) are in agreement with isolates found in milk-based products according to Hofer *et al.* (2000).

The presence of *Listeria monocytogenes* in the cheeses analyzed, points to the occurrence of failures in the cheese production process by some producers in the State of Paraná. The presence of other *Listeria* spp. species indicates that the production process may also permit contamination by *L. monocytogenes*, thus serving as an alert for a critical review of the process, to avoid contamination in critical points of the production process.

The absence of *Listeria monocytogenes* in the ice cream samples analyzed is supported by the fact that all the companies surveyed use UHT milk as a raw material for ice cream production. This result suggests that the work developed by the Sanitary Vigilance Agency of Paraná State is succeeding in the sense of providing guidance for the producers in the Good Fabrication Practices of 2002.

## CONCLUSION

Results show that the presence of *Listeria* spp. was not detected by the VIP<sup>®</sup> Biocontrol method in ice cream samples from the Curitiba region. In relation to the cheese samples sold in different regions of the State of Paraná, 6.70% of

them were inappropriate for consumption, according to the legal standard, because *Listeria monocytogenes* was present. In the other cheese samples, the isolation of *Listeria innocua* (5.50%) were also observed.

The existence of flaws in the production process of cheese produced in the State of Paraná is indicated by the occurrence of positive samples for *L. monocytogenes*. This illustrates the need for handling improvement during the collection and transportation of the raw material, and the processing of the cheese.

The results of the work show the viability of the use of the VIP method by the product's producers, thus, helping to reduce the risk of contaminated products reaching the consumer.

Effective action from the sanitary vigilance agency is required to preserve consumer health, due to the severity of hazardous infection which this microorganism can cause, especially to populations at risk.

## RESUMO

### Ocorrência de *Listeria monocytogenes* em queijos e sorvetes produzidos no Estado do Paraná, Brasil

A ocorrência de *Listeria monocytogenes* em sorvetes e alguns tipos de queijos macios e semi-macios produzidos e vendidos no Estado do Paraná Brasil foram avaliados. Noventa amostras de queijo e sessenta amostras de sorvete foram analisadas seguindo os protocolos da AOAC e FDA. Nas amostras de sorvete, não ocorreu o isolamento de *Listeria* spp. Foi detectada *Listeria* spp em 12,20% de amostras de queijo, das quais 6 (6,70%) foram positivas para *Listeria monocytogenes*. Foi também observada a presença de *Listeria innocua* em 5 amostras (5,50%) das mesmas amostras. Pelos resultados deste trabalho pode-se pressupor que existe um risco potencial à saúde da população brasileira com o agravante ainda, desta estar envelhecendo e aumentando o número de imunodeprimidos. Os resultados indicam a necessidade de implementação do monitoramento deste microrganismo pelos produtores bem como pela fiscalização. Mostra também que o método VIP é um método viável na triagem de amostras contaminadas para a liberação do produto para a comercialização por ser rápido, confiável e não requerer equipamentos adicionais.

**UNITERMOS:** *Listeria monocytogenes*/ocorrência. Queijo/análise microbiológica. Sorvete/análise microbiológica.

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