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Interference of raw milk autochthonous microbiota on the performance of conventional methodologies for *Listeria monocytogenes* and *Salmonella* spp. detection

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Summary

Pathogen detection in foods by reliable methodologies is very important to guarantee microbiological safety. However, peculiar characteristics of certain foods, such as autochthonous microbiota, can directly influence pathogen development and detection. With the objective of verifying the performance of the official analytical methodologies for the isolation of *Listeria monocytogenes* and *Salmonella* in milk, different concentrations of these pathogens were inoculated in raw milk treatments with different levels of mesophilic aerobes, and then submitted to the traditional isolation procedures for the inoculated pathogens. *Listeria monocytogenes* was inoculated at the range of 0.2–5.2 log CFU/mL in treatments with 1.8–8.2 log CFU/mL. *Salmonella* Enteritidis was inoculated at 0.9–3.9 log CFU/mL in treatments with 3.0–8.2 log CFU/mL. The results indicated that recovery was not possible or was more difficult in the treatments with high counts of mesophilic aerobes and low levels of the pathogens, indicating interference of raw milk autochthonous microbiota.

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This interference was more evident for *L. monocytogenes*, once the pathogen recovery was not possible in treatments with mesophilic aerobes up to 4.0 log CFU/mL and inoculum under 2.0 log CFU/mL. For *S.* Enteritidis the interference appeared to be more non-specific.

Introduction

Listeria monocytogenes and *Salmonella* spp. are pathogenic bacteria which frequently contaminate food products of animal origin, in particular those containing poultry, eggs, meat and milk (Boor, 1997; Jay et al., 2005). These pathogens can represent important hazards in the cited products, once several outbreaks and cases of food poisoning are associated with the consumption of contaminated dairy and meat products (de Buyser et al., 2001; Leclerc et al., 2002; Jay et al., 2005). Since their presence in these foods at low levels can pose a health risk (Bennett et al., 1998; Hof, 2003), it is important to recover even small concentrations of these bacteria through the recommended isolation methodologies (Beumer and Hazeleger, 2003).

The conventional methods for detection of *L. monocytogenes* and *Salmonella* spp. in foods are based in sequential steps of enrichment with selective culture media aiming to allow their growth, followed by their isolation using indicator culture media and identification of suspect colonies by biochemical characteristics (ISO, 1996, 2002; Wher and Frank, 2004; FDA, 2007). Some particularities of each pathogen are exploited in some methodologies, such as the ability of *L. monocytogenes* to multiply at low temperatures and its tolerance to specific antibiotics (ISO, 1996; Wher and Frank, 2004; Jay et al., 2005).

Particular characteristics of some foods can cause significant interferences in the recovery of *L. monocytogenes* and *Salmonella* spp. (Jay, 1995, 1996; de Boer, 1998). High microbial contamination in milk, usually observed in some regions (Nero et al., 2004; Kivaria et al., 2006; Adesiyun et al., 2007), and the presence of chemical substance residues (van Schaik et al., 2002) can inhibit the proper development of these pathogens during the isolation stages, and even in this food itself. These interferences could be considered as possible reasons for the great variance in pathogens prevalence in milk observed worldwide (Cordano and Rocourt, 2001; de Buyser et al., 2001; Leclerc et al., 2002; Nero et al., 2004; Jay et al., 2005).

Considering the importance of *L. monocytogenes* and *Salmonella* detection in milk, the objective of this study was to evaluate the performance of the officially recommended analytical methodologies for isolation of these pathogens in this food (Wher and Frank, 2004), verifying the interference caused by the autochthonous microbiota present in raw milk.

Material and methods

Bacterial strains and inoculations

Cultures of *L. monocytogenes* ATCC 7644 and *Salmonella* Enteritidis ATCC 13076, kept at 4 °C in Tryptone Soy Agar with 0.6% of Yeast Extract (TSA-YE, Oxoid, Basingstoke, Hampshire, UK) were streaked on plates with TSA-YE added to 7% defibrinated lamb blood and incubated for 24 h at 30 °C, for *L. monocytogenes*, and 35 °C, for *S.* Enteritidis. After incubation, some colonies of each microorganism were inoculated in Tryptone Soy Broth with Yeast Extract (0.6%) (TSB-YE, Oxoid), and incubated at the same temperatures initially used. After the inoculated TSA-YE became turbid (approximately 18 h), the pathogen concentration was estimated by absorbency at 660 nm (Cintra 5 UV visible spectrophotometer, GBC Scientific Equipment, Dandenong, VIC, Australia) and proper dilutions were carried out using 0.85% NaCl to obtain different pathogen concentrations for inoculation in the prepared treatments. Different dilutions were used for inoculation, to obtain a diversity of pathogen concentrations to be tested.

Raw milk samples and treatments

In a period of six weeks, six samples of raw milk were collected (one sample per week) from a dairy industry in sterile flasks and transported under refrigeration for laboratorial analysis (one hour maximum). Three samples were used to evaluate the performance of the *L. monocytogenes* isolation methodology and three for the *Salmonella* spp. isolation method. Initially, the samples were diluted twice to obtain different levels of contamination by the autochthonous microbiota, using sterile milk as diluent (10% reconstituted powder milk, Molico, Nestlé, São Paulo, Brazil). Different

dilution ratios were adopted for each sample, in order to obtain a wide variety of milk autochthonous microbiota concentration. Each raw milk sample and its two dilutions were then subdivided into four 100 mL aliquots, and artificially contaminated with the prepared cultures of *L. monocytogenes* and *S.* Enteritidis, at different concentrations of each pathogen. For each raw milk sample, the sterile milk used as diluent was used as a control and was also subdivided into four 100-mL aliquots that were inoculated with the pathogens. Consequently, each raw milk sample generated 12 treatments and further 4 controls, completing 16 tests as shown in Fig. 1.

The sterile milk controls inoculated with the pathogens were diluted serially with 0.85% NaCl. Considering the estimated quantity of pathogen inoculated, two dilutions were selected and plated on Petrifilm™ AC (3M Microbiology, St. Paul, MN, USA) and incubated at 35 °C for 48 h to determine the exact concentration of the pathogen.

Listeria monocytogenes detection

Immediately after inoculation, the treatments artificially contaminated with *L. monocytogenes* were submitted to the official detection methodology for this pathogen in raw milk (Wher and Frank, 2004), using culture medium from Oxoid. A 25 mL aliquot from each treatment or control was added to 225 mL of *Listeria* enrichment broth (LEB) that was incubated at 30 °C for 48 h. After incubation, one aliquot from each culture was streaked on Oxford agar and Palcam agar plates, and incubated at 35°C for 48 h. Typical *Listeria* spp. colonies were transferred to TSA-YE plates, incubated at 30 °C for 48 h and identified biochemically (Pagotto et al., 2007). The results were expressed as positive or negative for recovering *L. monocytogenes* in a 25 mL aliquot of each treatment or control.

Salmonella Enteritidis detection

Similar to the treatments inoculated with *L. monocytogenes*, immediately after inoculation, all the treatments artificially contaminated with *S.* Enteritidis cultures were submitted to the official detection methodology for this pathogen in raw milk (Wher and Frank, 2004), with a modification, using also culture media from Oxoid. A 25-mL aliquot of each treatment was added to 255 mL of lactose broth and after homogenization incubated at 35 °C for 24 h. After incubation, 0.1 mL was transferred to Rappaport–Vassilidadis broth – RV (incubated at 41 °C for 24 h) and simultaneously, 1.0 mL was transferred to Tetrationate broth – TT (incubated at 35 °C for 24-h). Selenite Cystine broth was substituted by RV broth due to its better performance in *Salmonella* spp. recovering (D'Aoust et al., 1992; Busse, 1995; de Boer, 1998). RV and TT broths were then streaked on Bismuth Sulfite agar, Xylose Lysine Desoxycholate agar and

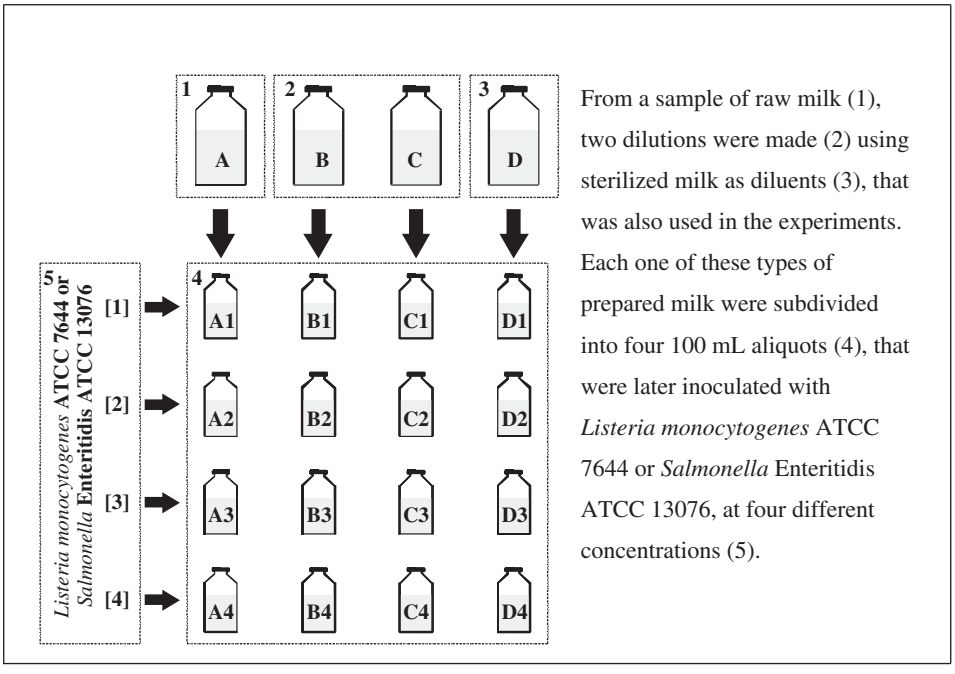

Figure 1. Scheme of the treatments preparation used in the evaluation of the performance of pathogen isolation in raw milk, *Listeria monocytogenes* and *Salmonella* spp.

Hektoen Enteric agar plates and incubated for 24 h at 35 °C. When suspect *Salmonella* colonies were present, they were transferred to Lysine Iron agar and Triple Sugar Iron agar slants, and incubated for 24 h at 35 °C. The results for *Salmonella* were confirmed through serological tests, using somatic and flagellar polyvalent antiserum (Probac do Brasil, São Paulo, SP, Brazil). The results were expressed as positive or negative for recovering *S.* Enteritidis in a 25 mL aliquot of each treatment or control.

Enumeration of mesophilic aerobes

Before the experimental inoculation, the raw milk samples and their dilutions in sterile milk were submitted to serial decimal dilutions in 0.85% NaCl. From the raw milk samples, the dilutions 1:100,000 and 1:1,000,000 were sown on Petrifilm™ AC plates to enumerate mesophilic aerobes, incubated at 35 °C for 48 h. Smaller dilutions of the other treatments were selected to enumerate these indicators.

Evaluation of inhibitor presence

All the raw milk samples used in this study were further tested regarding the presence of inhibitory substances of bacterial growth, using the Charm-Farm test™ FVN-100, following the manufacturer instructions (Charm Sciences Inc., Lawrence, MA, USA).

Results

The pathogen was recovered in all the treatments carried out with sterile milk artificially contaminated with *L. monocytogenes* or *S.* Enteritidis (the controls), regardless of the inoculum concentration.

Considering the different levels of contamination by the autochthonous microbiota, considerable variation in the recovery of the inoculated pathogens was observed, depending on the concentration of the cultures added experimentally (Figs. 2 and 3). Generally, a greater frequency of *L. monocytogenes* or *S.* Enteritidis recovering was observed in the treatments with low contamination levels of the autochthonous microbiota associated with higher inoculum concentrations of the pathogens.

The recovery of *L. monocytogenes* occurred in all treatments that presented concentrations higher than 2.2 log CFU/mL of the pathogen. In the treatments inoculated with 2.0 log CFU/mL of *L. monocytogenes*, pathogen recovery did not occur when the autochthonous microbiota contamination was higher than 5.0 log CFU/mL. At concentrations lower than 2.0 log CFU/mL of *L. monocytogenes*, pathogen recovery was only possible in the treatments that presented mesophilic aerobes at levels below to 4.0 log CFU/mL (Fig. 2).

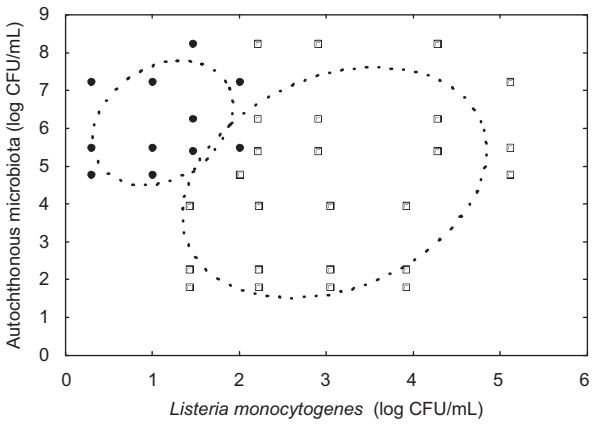

Figure 2. Positive (□) and negative results (●) for recovering of different levels of *Listeria monocytogenes* inoculated in raw milk treatments, with different levels of autochthonous microbiota. Ellipses indicate the range of positive and negative results at 0.95.

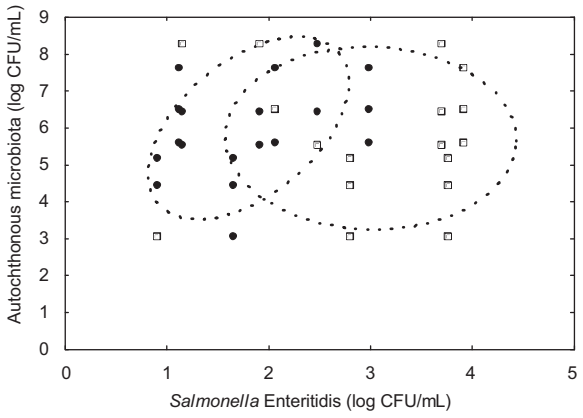

Figure 3. Positive (□) and negative results (●) for recovering of different levels of *Salmonella* Enteritidis inoculated in raw milk treatments, with different levels of autochthonous microbiota. Ellipses indicate the range of positive and negative results at 0.95.

The recovery of *S.* Enteritidis was possible in all treatments with concentrations higher than 3.7 log CFU/mL of the pathogen. In the treatments with 2.5–3.0 log CFU/mL of *S.* Enteritidis, the pathogen recovery was possible when the mesophilic aerobes were at levels below 5.5 log CFU/mL. When *S.* Enteritidis were inoculated at levels below 2.0 log CFU/mL, the pathogen was not recovered in any treatment, except in three cases: one treatment

that presented low level of pathogen (0.9 log CFU/mL) and autochthonous microbiota (3.1 log CFU/mL), and two treatments with relative low levels of the pathogen (1.2 and 1.9 log CFU/mL), associated with high levels of mesophilic aerobes (8.3 log CFU/mL) (Fig. 3). No sample contained residues of inhibitory substances for bacterial growth.

Discussion

The obtained results indicated that the tested methodologies for isolation of *L. monocytogenes* and *Salmonella* in milk were sensitive, without considering possible interfering factors of the samples. In all control treatments the recovery of the inoculated pathogen was possible. Pathogen isolation was possible even in the control treatments that were inoculated with the smallest concentrations of the pathogens. Different levels of pathogens and autochthonous microbiota were associated in order to verify possible interferences in the recovery methodologies. These pathogens are not usually quantified in dairy products, but often are present at low levels (0.0–2.0 log CFU/mL) and can represent risks to the consumers (Vlaemynck and Moermans, 1996; Hof, 2003; Jay et al., 2005).

The treatments with raw milk samples showed that pathogen recovery was not possible on several occasions, probably due to the intrinsic factors of the samples used. The possible interference by antimicrobial substances residues was discarded, since all samples presented negative results for this test. However, the autochthonous microbiota appears as determining factor for pathogen isolation, suggesting poor efficiency of the methodologies in these situations. The current microbiological standard for raw milk in Brazil is 1,000,000 CFU/mL of mesophilic aerobes (Farina et al., 2005), and at this level the recovery of *L. monocytogenes* was possible when the pathogens were present at high levels of contamination (Figs. 2 and 3).

That *Enterococcus* and *Lactobacillus* naturally occurring in milk interfere with the isolation and survival of *L. monocytogenes* was already described by Suh and Knabel (2001), and Jiang et al. (1998) demonstrated this interference during the enrichment phase of detection using LEB. In addition, *L. monocytogenes* is usually present in milk and dairy products at low levels (Vlaemynck and Moermans, 1996), similar to those simulated in the present study in treatments that showed no recovery of the pathogen. Regarding *S.* Enteritidis, we also observed a reduced recovery of the pathogen (Fig. 3), but this was not as evident as for *L. monocytogenes*. In a similar study, only using buffered peptone water instead of lactose broth in the initial phase, no significant interference was observed in the recovery of various concentrations of *Salmonella* added to raw milk containing mesophilic aerobes at levels of less than 3 log CFU/mL (Jiang et al., 1998). Some atypical and unexpected recovery results were observed (Fig. 3), suggesting differences in the microbial profile of the raw milk samples.

The difficulty of recovering *L. monocytogenes* and *Salmonella* in food has been reported, mainly in assessment studies of culture media and methodologies to isolate these microorganisms (D'Aoust et al., 1992; Vlaemynck and Moermans, 1996; Jiang et al., 1998; Suh and Knabel, 2001). The autochthonous microorganisms are the main interfering factor, since they can generate unfavorable conditions for the survival and detection of the pathogens (Busse, 1995; Vlaemynck and Moermans, 1996; Jiang et al., 1998; de Boer, 1998) and inhibit their growth by competition (Slade, 1992; Suh and Knabel, 2001). The multiplication of the autochthonous microbiota in the initial phases of *L. monocytogenes* and *Salmonella* isolation may cause a sharp fall in pH with consequent inhibition in the multiplication and detection of these pathogens. To avoid these interferences, alternative methods suggests the use of different culture media in these phases, such as Half-Fraser broth and Fraser broth for *L. monocytogenes* (ISO, 1996), and buffered Peptone Water and Selenite Cystine broth for *Salmonella* spp. (ISO, 2002). These variations aim to benefit the growth of these pathogens during the isolation procedures, especially for *L. monocytogenes* by using two selective media instead of only one (Wher and Frank, 2004), enhancing their recovery from raw milk samples.

Not only the concentration but also the composition the autochthonous microbiota in milk may interfere with the recovery of pathogens. Moreover, specific groups of microorganisms occurring naturally in milk are able to produce a great diversity of metabolites (Carr et al., 2002; Riley and Wertz, 2002) which can hinder the pathogen survival and growth (Jay, 1995, 1996; Besse, 2002). Milk also contains several antimicrobial substances that contribute to this interference, such as lactoferrin, whey proteins and casein fragments, bacteriophages and lacto-peroxidase system (Hudson et al., 2005; López-Expósito et al., 2006). All these substances, associated with specific storage and processing conditions, can also induce stress on *L. monocytogenes* and *Salmonella* spp., impairing their development and recovery. The treatments

were not submitted to any experimental stress or spoilage, which would certainly also affect recovery.

The results obtained led to the conclusion that the officially recommended analytical methodologies for *L. monocytogenes* and *Salmonella* detection in raw milk may suffer direct interference from the autochthonous microbiota in the samples to be analyzed. Further studies are necessary to clarify the underlying mechanisms. In addition, the development of more sensitive methods for *L. monocytogenes* and *Salmonella* detection in milk is recommended.

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