



Food Microbiology

Performance of two alternative methods for *Listeria* detection throughout Serro Minas cheese ripening



Gardênia Márcia Silva Campos Mata^a, Evandro Martins^a, Solimar Gonçalves Machado^a, Maximiliano Soares Pinto^b, Antônio Fernandes de Carvalho^b, Maria Cristina Dantas Vanetti^{a,*}

^a Department of Microbiology, Universidade Federal de Viçosa, Viçosa, MG, Brazil

^b Department of Food Technology, Universidade Federal de Viçosa, Viçosa, MG, Brazil

ARTICLE INFO

Article history:

Received 12 March 2015

Accepted 4 January 2016

Available online 22 April 2016

Associate Editor: Mariza Landgraf

Keywords:

Listeria

Raw milk cheese

Alternative methods

ABSTRACT

The ability of pathogens to survive cheese ripening is a food-security concern. Therefore, this study aimed to evaluate the performance of two alternative methods of analysis of *Listeria* during the ripening of artisanal Minas cheese. These methods were tested and compared with the conventional method: *Lateral Flow System*TM, in cheeses produced on laboratory scale using raw milk collected from different farms and inoculated with *Listeria innocua*; and VIDAS[®]-LMO, in cheese samples collected from different manufacturers in Serro, Minas Gerais, Brazil. These samples were also characterized in terms of lactic acid bacteria, coliforms and physical–chemical analysis. In the inoculated samples, *L. innocua* was detected by *Lateral Flow System*TM method with 33% false-negative and 68% accuracy results. *L. innocua* was only detected in the inoculated samples by the conventional method at 60-days of cheese ripening. *L. monocytogenes* was not detected by the conventional and the VIDAS[®]-LMO methods in cheese samples collected from different manufacturers, which impairs evaluating the performance of this alternative method. We concluded that the conventional method provided a better recovery of *L. innocua* throughout cheese ripening, being able to detect *L. innocua* at 60-day, aging period which is required by the current legislation.

© 2016 Sociedade Brasileira de Microbiologia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Artisanal cheeses are widely appreciated and constitute a specific group of cheeses produced on farms on a small scale using traditional techniques.¹ In addition to their cultural, social and economic relevance, these cheeses also have a complex

microbial ecosystem associated with raw milk, cattle management and changes that occur in this food matrix during ripening, which contribute to the unique sensory characteristics of this product.^{2–5} Traditional Brazilian cheese includes varieties classified according to their region in Minas Gerais, and the most important varieties are produced in Serro, Canasta, Cerrado and Araxá.^{5–7} Serro Minas cheese is usually

* Corresponding author.

E-mail: mvanetti@ufv.br (M.C.D. Vanetti).

<http://dx.doi.org/10.1016/j.bjm.2016.04.006>

1517-8382/© 2016 Sociedade Brasileira de Microbiologia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

made from raw bovine milk with addition of the “pingo”, a natural fermentation starter originated from whey collected from successful cheese production from the previous batch.⁸

There is a clear risk of pathogen transmission in the production of artisanal cheese.⁹ Loncarevic et al.,¹⁰ for example, found *Listeria monocytogenes* in 42% of cheeses made from raw milk and in 2% of cheeses made from pasteurized milk. *L. monocytogenes* is a Gram-positive bacteria and causal agent of listeriosis whose clinical symptoms may include gastrointestinal diseases, meningitis, septicemia or even death.¹¹

In industrialized countries, milk and dairy products are involved in 2–6% of outbreaks of foodborne illnesses¹² and *L. monocytogenes* is one of the major pathogens involved in these outbreaks.¹³ Throughout the world, 261 clinical cases and 18 deaths were caused by listeriosis outbreaks associated with raw milk or raw milk cheese from January 2000 to 2010.¹⁴ Annually, *L. monocytogenes* is responsible for approximately 2500 cases of listeriosis, 2289 hospitalizations and 449 deaths in the United States.¹⁵

To avoid illnesses in the consumption of artisanal cheeses, it is recommended in addition to the adoption of Good Manufacturing Practices and Hazard Analysis and Critical Control Point tools¹⁶ that the cheeses be aged for 60 days prior to commercialization.^{17,18} Brazilian law was recently changed, thus allowing raw milk cheeses be matured for a period less than 60 days, if the provided technical and scientific studies demonstrate that reducing the maturation period does not compromise the quality and safety of the product.¹⁹ This rule is based on the assumption that even if pathogenic microorganisms were initially present in raw milk, they would be inactivated by changes throughout ripening,²⁰ which include low pH, water activity, high salt content and a competitive environment.²¹ However, studies suggest that if pathogenic bacteria are present in the milk prior to cheese production, they could still survive.^{22–24} Safe *L. monocytogenes* levels can vary until 100 CFU/g, only for products where the growth of *L. monocytogenes* is maintained in this limit until the end of its shelf life,²⁵ to absent in 25 g.^{26,27}

The current legislation on food and health suggests an increased need for sample collection and analytical methods that are faster, cost-effective and easy to apply in the industry.^{26,28} Therefore, alternative pathogen detection methods in food have proven to be positive for the industry because of their practicality, agility and potential for automation.²⁹ These methods eliminate some steps relative to conventional methodologies, such as selection of typical colonies on selective culture media and morphological, biochemical and serological tests.²⁸ Current molecular methods based on the amplification of target DNA by PCR and immunodetection based on the antigen–antibody reaction are the main alternative methods for pathogen detection.^{30–34} The analytical methods must also be suited to the food matrix and have good performance attributes such as a low detection limit and high sensitivity, specificity and accuracy. Emphasis is given to the adequacy of the pathogen detection methods to the intrinsic feature of the food matrix, since the competing microbiota³⁵ and physical–chemical can interfere with performance of these methods. So here, we showed a study comparing the performance of two alternative methods of analysis of *Listeria* against the conventional method

throughout artisanal Minas cheese ripening, also taking into account the influence of the intrinsic characteristics of these samples in the analyses.

Materials and methods

Detection of *L. innocua* by the conventional and immunoanalytical methods in artificially contaminated artisanal Minas cheese samples

Fifteen artisanal Minas cheese samples were produced on laboratory scale from raw milk obtained from three suppliers in the Serro region and was artificially contaminated with 10 CFU/mL of *L. innocua* ATCC 33090 as a surrogate for *L. monocytogenes*. The cheese samples were manufactured as described by Pinto et al.²³ Negative controls were also produced with raw milk not inoculated with *L. innocua*.

The survival of *L. innocua* was evaluated using conventional and immunoanalytical methods at five different times of ripening (5, 15, 30, 45 and 60 days). In each period, three independent samples were evaluated.

To detect *L. innocua* using conventional method,³⁶ 25 g of the cheese were homogenized in 225 mL of *Listeria* Enrichment Broth – LEB (Acumedia, Lansing, USA), and after incubation for 20–24 h at 30 ± 1 °C, 0.1 mL aliquots were transferred to 10 mL of supplemented Fraser broth (Oxoid, Basingstoke, UK). After incubation for 25 ± 1 h at 30 ± 1 °C, selective plating was performed in Oxford agar (Difco, Sparks, USA) and Palcam agar (Merck, Darmstadt, Germany). Typical *Listeria* sp. colonies were selected on TSA agar (Oxoid) containing 6% (w/v) yeast extract (MicroMed, Rio de Janeiro, Brazil) and submitted to biochemical characterization. Biochemical tests included catalase, Gram stain, motility, nitrate reduction, methyl red, Voges Proskauer, carbohydrate fermentation in phenol-red broth with xylose (Vetec, Rio de Janeiro, Brazil), rhamnose (Merck), mannitol (Merck) and alpha-hemolysis in Columbia agar (Oxoid) supplemented with 5% (v/v) defibrinated sheep blood.

The immunoassay method *Listeria* Test Kit PN 18220002 DuPont™ Lateral Flow System™ (DuPont Qualicon, Wilmington, USA) was also used to detect *Listeria* sp. in the same samples previously described, according to the manufacturer's recommendations. Aliquots of the enrichment broth were boiled in a water bath for 15 min, transferred to microtubes containing immobilized anti-*Listeria* sp. antibodies and then the results were read after 10 min at room temperature.

Detection of *L. monocytogenes* by the conventional and immunoanalytical methods in artisanal Minas cheese samples

A total of 48 samples of Serro Minas cheese with different ripening times were collected from different manufacturers in Serro, Minas Gerais, Brazil. Half of these samples had ripening times less than 60 days and the other samples were greater than 60 days. The analysis of *L. monocytogenes* was performed according to the conventional method described above. To detect *L. monocytogenes* by the VIDAS®-LMO method, from bioMérieux, Marcy l'Etoile, France,³⁷ 25 g of cheese

samples were homogenized in 225 mL of supplemented Fraser broth (Oxoid). After incubation for 25 ± 1 h at 30 ± 1 °C, 1 mL aliquots were transferred to 10 mL of Fraser broth without any supplement. After incubation under the same conditions, 1 mL aliquot of the secondary enrichment broth was boiled for 15 min, and 0.5 mL of this suspension was analyzed in the Mini-VIDAS® immunoassay analyzer (bioMérieux, Marcy l'Etoile, France) using the VIDAS®-LMO kit.

Evaluation of intrinsic characteristics of artisanal Minas cheese

The intrinsic characteristics of all artisanal Minas cheese samples were evaluated in terms of the lactic acid bacteria count, enumeration of total and thermotolerant coliforms and physical–chemical analysis.

Samples of 25 g were homogenized in 225 mL of saline peptone 0.1% (w/v) then the decimal dilutions were made and the most appropriate one was used to carry out two analyses. In the first, the aliquots were inoculated in MRS agar (Acumedia) with the pH indicator bromocresol purple (Merck) to assess the production of acid compounds. The plates were incubated in anaerobic jars (Oxoid) in a microaerophilic environment and incubated at 30 °C for 72 ± 3 h. Additional tests such as the Gram stain and catalase were performed to confirm the lactic acid bacteria count in the samples.³⁸ In the second, the aliquots were also transferred to series of three tubes for incubation at 36 ± 1 °C in lauryl sulfate tryptose (LST) broth (Merck) for the presumptive coliform test and Brilliant Green Broth (Merck) for the total coliforms confirmatory test. EC broth (Acumedia), incubated at 45 °C was used to confirm the presence of thermotolerant coliforms.³⁸

The water activity (a_w) was measured in an automatic analyzer (Decagon Aqualab, CX-2, Washington, USA). The pH was determined with a pHmeter (Tecnopeon MPA-210P, São Paulo, Brazil) according to Richardson,³⁹ as were the titratable acidity and NaCl content. The moisture content was determined as the ratio between weight loss of the samples after drying at 102 ± 2 °C for 3 h and the initial weight of 5 g.

Data analysis

In order to avoid season variability, all artisanal Minas cheese samples were made or collected in the rainy season (October–March). The results of the microbiological evaluation

Equation 1:

$$\text{Sensitivity} = \frac{(\text{Positive samples by the conventional method} - \text{false negatives}^*) \times 100}{\text{Total samples positive by the conventional method}}$$

Equation 2:

$$\text{Specificity} = \frac{(\text{Negative samples by the conventional method} - \text{false positives}^{**}) \times 100}{\text{Total samples positive by the conventional method}}$$

Equation 3:

$$\text{Accuracy} = \frac{(\text{Positive samples by the conventional method} - \text{false negatives}) + (\text{Negative samples by the conventional method} - \text{false positives}) \times 100}{\text{Total samples}}$$

* False negative: negative samples by the alternative method but positive by the conventional method.
 ** False positive: positive samples by the alternative method but negative by the conventional method.

Fig. 1 – Equations used for analysis of the performance of rapid methods of detection of *Listeria* in the artisanal Minas cheese samples.

were expressed as presence or absence in 25 g for *Listeria* sp. or *L. monocytogenes* detection, in log CFU/g for lactic acid bacteria count and in log MPN/g for coliform counts. The Lateral Flow System™ and VIDAS®-LMO methods were compared to the conventional method and the performance of both methods was evaluated in terms of sensitivity, specificity and accuracy results. The analyses were performed using the Epi Info software.⁴⁰ The following equations were used (Fig. 1).

A descriptive statistical analysis was also used to characterize the samples in terms of physico-chemical characteristics and endogenous microbiota. The ANOVA test and the Tukey post-test or the Student's t-test were used to evaluate the differences between the mean of the parameters mentioned above, considering the ripening time of the cheese samples.

Results

Evaluation of the performance of Lateral Flow System™ and VIDAS®-LMO methods

Out of 15 artisanal Minas cheese samples artificially contaminated with *L. innocua* analyzed using Lateral Flow System™ method, a total of approximately 54% were positive for *Listeria* against approximately 87% of positive samples detected by the conventional method. The Lateral Flow System™ method showed a poor performance for this food matrix when compared to the conventional method due to the low sensitivity and accuracy values (Table 1). Our results showed a high discrepancy between sensitivity and specificity values in the Lateral Flow System™ method. The low sensitivity value demonstrates a high detection limit and a low sensitivity to the antibody used. This observation is confirmed by the considerable proportion of approximately 34% of false-negative results (Table 1).

The intentional contamination of raw milk allowed evaluating the survival of *L. innocua* over the ripening period of the artisanal cheese samples. By the conventional method, *L. innocua* could be recovered throughout all ripening days.

Table 1 – Performance of the Lateral Flow System™ method for the detection of *Listeria* in artisanal Minas cheese samples.

Number of samples	Lateral Flow System™
Tested	15
Positive by the conventional method	13
Positive by the alternative method	8
Negative by the conventional method	2
Negative by the alternative method	7
False positive	0
False negative	5
Sensitivity ^a (%)	61.54
Specificity ^b (%)	100.00
Accuracy ^c (%)	66.67

^a Equation 1.

^b Equation 2.

^c Equation 3 (Please, see this equations which are placed on Fig. 1 in Materials and Methods section).

Table 2 – Survival of *L. innocua* in artisanal Minas cheese samples produced in Serro, Brazil, over 60 days of ripening.

Test results	Ripening time (days)				
	5	15	30	45	60
Conventional	3/3 ^a	3/3	3/3	3/3	1/3
Lateral Flow System™	1/3	3/3	2/3	2/3	0/3

^a Positive samples/total samples.

However, by Lateral Flow System™ method *L. innocua* could be recovered up until 45 days, but not at 60 days of ripening (Table 2).

L. monocytogenes was not detected in the Serro Minas cheese samples obtained directly from producers and analyzed at different ripening times by either the conventional or VIDAS®-LMO methods. The low frequency of *L. monocytogenes* in Serro Minas cheese samples hinders the efficient assessment of the performance of the method since the sensitivity is null and specificity is 100%.

Intrinsic characteristics of artisanal Minas cheeses

There was a wide variation in the intrinsic characteristics in the cheese samples throughout ripening. Some of these

analyses showed statistical differences between the ripening times (Tables 3 and 4). These analyses should be evaluated carefully, as they may interfere with the *Listeria* growth and consequently with the performance of the analysis methods.

The artificially contaminated samples with *L. innocua* presented lactic acid bacteria, total coliform and thermo-tolerant coliform counts above 6 log CFU/g, 3 log MPN/g and 2 log MPN/g, respectively (Tables 3 and 4). Only the coliform group reduced throughout the days of ripening (Tables 3 and 4). The high counts of total and thermotolerant coliforms is an indication of low hygiene quality of the raw material used in the manufacturing of this cheese and it is important to consider that the ripening over 60 days was important for their reduction to safe levels, according to current legislation which requires values less than 10⁴ total coliforms/g of cheese and 5 × 10³ thermotolerant coliforms/g of cheese.

The low pH and high titratable acid values at the end of ripening reflect the lactic acid produced by lactic acid bacteria (Tables 3 and 4). The variation in the NaCl content in the samples during the cheese ripening (Table 3) may reflect the lack of standardization of salting during the artisanal manufacturing process of these cheeses. The water activity is reduced throughout cheese ripening due to the loss of moisture and consequently, the NaCl content increased in samples collected from different manufacturers (Table 4).

Table 3 – Changes in the intrinsic characteristics of the artisanal Minas cheese samples^b artificially contaminated with *L. innocua* produced in Brazil throughout ripening.

Parameters	Days of ripening				
	5	15	30	45	60
Lactic acid bacteria (log CFU/g)	7.40 ± 0.56 ^a	7.43 ± 0.23 ^a	7.05 ± 0.27 ^a	6.67 ± 0.40 ^a	6.65 ± 0.65 ^a
Total coliforms (log MPN/g)	5.44 ± 0.64 ^a	3.71 ± 0.33 ^{ac}	2.23 ± 0.30 ^{bc}	2.60 ± 0.22 ^{bc}	1.14 ± 0.76 ^b
Thermotolerant coliforms (log MPN/g)	4.80 ± 0.35 ^a	2.48 ± 0.00 ^b	0.99 ± 0.34 ^c	0.83 ± 0.25 ^c	0.48 ± 0.00 ^c
Water activity (<i>a_w</i>)	0.910 ± 0.002 ^a	0.896 ± 0.006 ^a	0.867 ± 0.012 ^{ab}	0.868 ± 0.008 ^{ab}	0.831 ± 0.020 ^b
Moisture (%)	42.810 ± 0.263 ^a	34.831 ± 1.186 ^b	30.8442 ± 1.567 ^{bd}	31.5717 ± 0.997 ^{bd}	28.5437 ± 1.019 ^{cd}
pH	5.493 ± 0.044 ^{ab}	5.780 ± 0.052 ^c	5.613 ± 0.038 ^b	5.533 ± 0.052 ^a	5.390 ± 0.033 ^a
Titratable acidity (lactic acid %)	1.004 ± 0.062 ^a	0.948 ± 0.059 ^a	1.228 ± 0.022 ^a	1.348 ± 0.127 ^a	1.423 ± 0.178 ^a
NaCl in moisture (%)	0.938 ± 0.029 ^a	0.539 ± 0.080 ^b	0.559 ± 0.049 ^b	0.563 ± 0.053 ^b	0.691 ± 0.073 ^{ab}

^b Data are the average values and standard error of three batches. There is no statistical difference between the means of the parameters followed by at least the same letter considering the days of ripening at 5% probability by Tukey test.

Table 4 – Changes in the intrinsic characteristics of Serro Minas cheese samples^c collected from different manufacturers in Serro, Brazil, are grouped in two ripening times.

Parameters	Values	
	<60 days of ripening (n = 24)	>60 days of ripening (n = 24)
Lactic acid bacteria (log CFU/g)	7.98 ± 0.15 ^a	6.71 ± 0.17 ^a
Total coliforms (log MPN/g)	3.02 ± 0.27 ^a	0.85 ± 0.14 ^b
Thermotolerant coliforms (log MPN/g)	2.3 ± 0.22 ^a	0.68 ± 0.10 ^b
Water activity (<i>a_w</i>)	0.913 ± 0.001 ^a	0.866 ± 0.009 ^b
Moisture (%)	50.832 ± 0.978 ^a	38.423 ± 1.915 ^b
pH	5.420 ± 0.081 ^a	5.822 ± 0.133 ^b
Titratable acidity (lactic acid %)	0.957 ± 0.045 ^a	1.070 ± 0.087 ^b
NaCl in moisture (%)	0.849 ± 0.085 ^a	0.855 ± 0.049 ^b

^c Data are the average values and standard error of 24 batches. There is no statistical difference between the means of the parameters followed by at least the same letter considering the days of ripening at 5% probability by Student's t-test.

Discussion

In this study, we evaluated the survival of *L. innocua* during artisanal cheeses ripening by using two analytical methods. The applicability of the use of *L. innocua* instead of *L. monocytogenes* has been reviewed previously and even in other cheese studies. Furthermore, *L. innocua* has also been isolated from artisanal cheeses. These strains are physiologically close, so *L. innocua* is an effective biological indicator of the potential survival of *L. monocytogenes*.^{41–47} Regarding this, the intentional contamination of raw milk cheese with *L. innocua* provided a better comparison between the conventional and Lateral Flow System™ methods due to the increase of the frequency of the evaluated microorganism. The low sensitivity by the Lateral Flow System™ method indicates the difficulty of this method in discriminating results which are positive. The low accuracy values indicate the difficulty of using this method in discriminating positive results when the pathogen is present and also for negative results when the pathogen is absent (Table 1). Moreover, a good method should have both high and similar sensitivity and specificity values because these factors would yield fewer false-positive and false-negative results and therefore provide high accuracy.

The intentional contamination of the samples with *L. innocua* also allows the evaluation of survival of this bacterium throughout the ripening of artisanal Minas cheese (Table 2). Therefore, *L. monocytogenes* would be able to survive for 60 days of ripening in the artisanal Minas cheese. In agreement with our findings, the survival of *L. monocytogenes* was confirmed at 42 days of ripening in cheeses made with raw goat's milk, a traditional French cheese and in cheeses inoculated with 10² CFU/mL of this pathogen.²² Rogga et al.⁴⁸ observed that the type of cheese (industrial or artisanal) and the storage temperature did not significantly affect the survival of *L. monocytogenes* inoculated at 10³ CFU/mL. In a traditional cheese from Portugal made from raw sheep's milk, a significant increase in the *L. monocytogenes* count was observed over 42 days of ripening.⁴⁹ The initial *L. monocytogenes* contamination at 10³ CFU/mL or 10⁷ CFU/mL did not significantly affect the number of pathogens that survived in Galotyri industrial cheese ripened for 28 days and stored at 4°C.⁴⁸ The number of *L. monocytogenes* increased during the manufacturing and ripening of Camembert-type cheese made from raw cow's milk.⁵⁰ Pinto et al.²³ showed that *L. innocua* can grow throughout the ripening of artisanal cheeses made with cow's milk, and the intrinsic characteristics of the cheese apparently did not interfere with detection by the conventional method.

Our results showed by conventional and VIDAS®-LMO methods, the absence of *L. monocytogenes* in Serro Minas cheese samples obtained directly from producers and, considering this, these samples attend specifications established by Brazilian law,²⁷ which is the absence of *L. monocytogenes* in 25 g of cheese. The absence of *L. monocytogenes* from cheeses made from raw milk in the United States and Brazil was also reported by Brooks et al.²¹ and Galinari et al.,⁵¹ respectively. However, these results must be viewed with caution, since we must consider that contamination of these samples might be low or there may be inhibition of *L. monocytogenes* throughout the ripening, since this pathogen is resistant to adverse

conditions and can survive in the ripened cheese.⁵² In other studies this pathogen has been detected in artisanal cheeses^{14,53,54} and cheeses made from raw milk have been reported as vehicles in listeriosis outbreaks.^{14,55}

Despite of our negative results, the efficiency of the VIDAS®-LMO method has been reported in other food matrices, such as ice cream, cheese, cooked roast beef, frozen green beans and frozen tilapia. Of the 457 positive samples detected by the conventional method, 448 were positive by the VIDAS®-LMO method, and there was no significant difference between the methods.⁵⁶ The VIDAS®-LIS method showed 86% concordance with the conventional culture method for the detection of *Listeria* sp. in food. Of the 935 positive samples, 809 were detected by the conventional method and 839 by the VIDAS®-LIS method.⁵⁷ In meat samples, a high number of false-positive results for *Listeria* using the VIDAS® system was found in a previous study;⁵⁸ this method was more suitable for detecting negative samples.

Although it was not possible to properly evaluate the performance of the VIDAS®-LMO method in this study, other studies in the literature have shown promising results with respect to this method. The VIDAS® method yielded values of 98.1%, 97.0% and 97.5%, for sensitivity, specificity and accuracy, respectively. The rates of false negatives and false positives were 1.9% and 3.0%, respectively, for the detection of *L. monocytogenes* in food samples.⁵⁹ Conflicting results are also reported in the literature regarding the performance of immunoanalytical methods. Aldus et al.⁶⁰ observed good performance of the Lateral Flow immunoassay for detection of verotoxigenic *Escherichia coli*, with a false-negative rate of less than 2%, a false-positive rate between 9 and 6% and a detection limit of 3 CFU/g, also meeting the Canadian criteria⁶¹ for alternative methods.

Cheeses made from raw milk have heterogeneous microbiota and the population of lactic acid bacteria reaches numbers above 6 log CFU/g (Tables 3 and 4). This microbiota, which were present in concentrations under 6 log MPN/g, is essential to inhibit the growth of undesirable microorganisms such as coliforms. Even as in the study of Borelli et al.⁶² and Cardoso et al.⁶³ the ripening process of our samples was effective in reducing the contamination detected by the most important microbiological indicators for contamination of cheese according to Brazilian law.

The presence of competing microbiota (Table 3) in artisanal cheese may have another effect, which is the compromised detection of *Listeria* when this bacteria is present (Table 2). Maybe this is why we observed a performance difference between the two methods used to evaluate the artificially contaminated cheese samples. The interference of the endogenous microbiota of raw milk in the detection of *L. monocytogenes* by the conventional methodology has been demonstrated by Nero et al.³⁵ These authors observed that the recovery of *L. monocytogenes* at concentrations below 2 log CFU/mL was possible only when the endogenous microbiota was present at concentrations below 4 log CFU/mL. Imran et al.⁶⁴ also demonstrated, using culture and mathematical modeling, that the competitor community significantly reduces the growth of *L. monocytogenes*, regardless of the pH. It is likely that the combined effect of the

endogenous competing microbiota which produce inhibitory compounds such as organic acids, hydrogen peroxide and bacteriocins and the physical-chemical characteristics of artisanal cheeses injure pathogenic cells and affect the performance of the methods evaluated.^{11,12,65}

Some physical-chemical results (Tables 3 and 4) were in accordance with Souza et al.⁶⁶ These intrinsic characteristics changes (Tables 3 and 4) throughout cheese ripening could inhibit the growth of *Listeria* and other pathogens if present but not necessarily viability loss. Because of this, the fundamental focus on avoiding the public risks of these products is the adoption of good manufacturing practices.

Conclusions

This study showed that the conventional method provided a better recovery of *L. innocua* throughout artisanal Minas cheese ripening in artificially contaminated samples than immuno-analytical methods, which is probably due to the interference of the intrinsic characteristics of the artisanal cheeses. The intentional contamination of cheeses with *L. innocua* also demonstrated that this microorganism is able to survive during the ripening period required by legislation. So, although the alternative methods for detection of microorganisms in food are beneficial mainly because they obtain faster results, their performance should be evaluated for applicability to the food matrix. Despite the low frequency of *L. monocytogenes* in Serro Minas cheese, which impairs evaluating the performance of the method VIDAS®-LMO, cheeses made with raw milk offer a potential health risk to consumers. Thus, the use of quality tools such as good practices should be a subject of attention in this type of product.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgment

The authors would like to thank to FAPEMIG for financial support.

REFERENCES

- Randazzo CL, Caggia C, Neviani E. Application of molecular approaches to study lactic acid bacteria in artisanal cheeses. *J Microbiol Methods*. 2009;78:1–9.
- Beuvier E, Buchin S. Raw milk cheeses. In: Fox PF, McSweeney PLH, Cogan TM, Guinee TP, eds. *Cheese: Chemistry, Physics and Microbiology. General Aspects*. vol. 1, 3rd ed. London: Elsevier Academic Press; 2004:319–345.
- Renye JA Jr, Somkuti GA, Vallejo-Cordoba B, Van Hekken DL, Gonzalez-Cordova AF. Characterization of the microflora isolated from queso fresco made from raw and pasteurized milk. *J Food Saf*. 2008;28:59–75.
- Mallet A, Guéguen M, Kauffmann F, Chesneau C, Sesboué A, Desmasures N. Quantitative and qualitative microbial analysis of raw milk reveals substantial diversity influenced by herd management practices. *Int Dairy J*. 2012;27:13–21.
- Arcuri EF, El Sheikh AF, Rychlik T, Piro-Métayer I, Montet D. Determination of cheese origin by using 16S rDNA fingerprinting of bacteria communities by PCR-DGGE: preliminary application to traditional Minas cheese. *Food Control*. 2013;30:1–6.
- Alexandre DP, Silva MR, Souza MR, Santos WLM. Atividade antimicrobiana de bactérias lácticas isoladas de queijo-de-Minas artesanal do Serro (MG) frente a micro-organismos indicadores. *Arq Bras Med Vet Zootec*. 2002;54:424–428.
- Borelli BM, Ferreira EG, Lacerda ICA, Franco GR, Rosa CA. Yeast populations associated with the artisanal cheese produced in the region of Serra da Canastra, Brazil. *World J Microb Biot*. 2006;22:115–1119.
- Cardoso VM, Borelli BM, Lara CA, et al. The influence of seasons and ripening time on yeast communities of a traditional Brazilian cheese. *Food Res Int*. 2015;69:331–340.
- Omiccioli E, Amagliani G, Brandi G, Magnani M. A new platform for real-time PCR detection of *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157 in milk. *Food Microbiol*. 2009;26:615–622.
- Loncarevic S, Danielsson-Tham ML, Tham W. Occurrence of *Listeria monocytogenes* in soft and semi-soft cheeses in retail outlets in Sweden. *Int J Food Microbiol*. 1995;26:245–250.
- Sip A, Więckowicz M, Olejnik-Schmidt A, Grajek W. Anti-*Listeria* activity of lactic acid bacteria isolated from golka, a regional cheese produced in Poland. *Food Control*. 2012;26:117–124.
- Claeys WL, Cardoen S, Daube G, et al. Raw or heated cow milk consumption: review of risks and benefits. *Food Control*. 2013;31:251–262.
- Kousta M, Mataragas M, Skandamis P, Drosinos EH. Prevalence and sources of cheese contamination with pathogens at farm and processing levels. *Food Control*. 2010;21:805–815.
- Hall WF, French N. *An Assessment of Available Information on Raw Milk Cheeses and Human Disease 2000–2010*. Wellington: Ministry of Agriculture and Forestry; 2011.
- Center of Disease Control (CDC). *Estimates of Foodborne Illness in the United States 2011; 2011*. Accessed 04.07.11.
- Kramarenko T, Roasto M, Meremäe K, Kuningas M, Põltsama P, Elias T. *Listeria monocytogenes* prevalence and serotype diversity in various foods. *Food Control*. 2013;30:24–29.
- Brasil. Ministério da Agricultura, Pecuária e Abastecimento. Portaria n° 146 de 7 de março de 1996. Regulamento técnico de identidade e qualidade de queijos. Diário Oficial da República Federativa do Brasil. Brasília 11 March 1996.
- Food and Drug Administration. *Code of Federal Regulations, Title 21 and Food and Drugs, Part 133 Cheeses and Related Cheese Products*. Washington, DC; 2011. Accessed 13.08.15.
- Brasil. Ministério da Agricultura, Pecuária e Abastecimento. Instrução Normativa n° 30, de 7 de agosto de 2013. Diário Oficial da República Federativa do Brasil. Brasília 08 August 2013.
- Wu VCH. A review of microbial injury and recovery methods in food. *Food Microbiol*. 2008;25:735–744.
- Brooks JC, Martinez B, Stratton J, Bianchini A, Krokstrom R, Hutkins R. Survey of raw milk cheeses for microbiological quality and prevalence of foodborne pathogens. *Food Microbiol*. 2012;31:154–158.
- Morgan F, Bonnin V, Mallereau MP, Perrin G. Survival of *Listeria monocytogenes* during manufacture, ripening and storage of soft lactic cheese made from raw goat milk. *Int J Food Microbiol*. 2001;64:217–221.
- Pinto MS, Carvalho AF, Pires ACS, Paula JJC, Sobral D, Magalhães FAR. Survival of *Listeria innocua* in Minas

- Traditional Serro cheese during ripening. *Food Control*. 2009;20:1167–1170.
24. Schwartzman MS, Maffre A, Tenenhaus-Aziza F, Sanaa M, Butler F, Jordan K. Modelling the fate of *Listeria monocytogenes* during manufacture and ripening of smeared cheese made with pasteurised or raw milk. *Int J Food Microbiol*. 2011;145(suppl 1):31–38.
 25. Commission of the European Communities. *Commission Regulation (EC) n° 2073/2005 of 15 November 2005 on Microbiological Criteria for Foodstuffs*; 2005. Accessed 04.07.11.
 26. Schirmer BC, Langsrud S, Moretro T, Hagtvædt T, Heir E. Performance of two commercial rapid methods for sampling and detection of *Listeria* in small-scale cheese producing and salmon processing environments. *J Microbiol Methods*. 2012;91:295–300.
 27. Brasil. Ministério da Saúde Agência Nacional de Vigilância Sanitária. Resolução n°. 12 de 10 de Janeiro de 2001. Regulamento Técnico sobre padrões microbiológicos para alimentos. Diário Oficial da República Federativa do Brasil. Brasília 10 January 2001.
 28. Velusamy V, Arshak K, Korostynska O, Oliwa K, Adley C. An overview of foodborne pathogen detection: in the perspective of biosensors. *Biotechnol Adv*. 2010;28:232–254.
 29. Olsen JE. DNA-based methods for detection of food-borne bacterial pathogens. *Food Res Int*. 2000;33:257–266.
 30. Jadhav S, Bhavne M, Palombo EA. Methods used for the detection and subtyping of *Listeria monocytogenes*. *J Microbiol Methods*. 2012;88:327–341.
 31. Jasson V, Jacxsens L, Luning P, Rajkovic A, Uyttendaele M. Alternative microbial methods: an overview and selection criteria. *Food Microbiol*. 2010;27:710–730.
 32. Machado SG, Bazzolli DMS, Vanetti MCD. Development of a PCR method for detecting proteolytic psychrotrophic bacteria in raw milk. *Int Dairy J*. 2013;29:8–14.
 33. Mata GMSC, Vanetti MCD. Comparison of conventional and rapid methods for *Salmonella* detection in artisanal Minas cheese. *J Food Res*. 2012;1:178–183.
 34. Zunabovic M, Domig KJ, Kneifel W. Practical relevance of methodologies for detecting and tracing of *Listeria monocytogenes* in ready-to-eat foods and manufacture environments – a review. *LWT – Food Sci Technol*. 2011;44:351–362.
 35. Nero LA, Mattos MR, Barros MA, Beloti V, Franco BD. Interference of raw milk autochthonous microbiota on the performance of conventional methodologies for *Listeria monocytogenes* and *Salmonella* spp. detection. *Microbiol Res*. 2009;164:529–535.
 36. Andrews WH, Flowers RS, Silliker J, Bailey JS. *Salmonella*. In: Downes FP, Ito K, eds. *Compendium of Methods for the Microbiological Examination of Foods*. 4th ed. Washington: American Public Health Association; 2001:357–380.
 37. Andrews WH, Hammack TS. Microbiological methods. AOAC Official Method 2004.02 *Listeria monocytogenes* in selected foods. VIDAS *Listeria monocytogenes* II (LMO2). In: Howtitz W, Latimer GW, eds. *Official Methods of Analysis of AOAC International*. 18th ed. Maryland: AOAC International; 2006:233–234.
 38. Downes FP, Ito K, eds. *Compendium of Methods for the Microbiological Examination of Foods*. Washington: American Public Health Association; 2001.
 39. Richardson GH. *Standard Methods for the Examination of Dairy Products*. Washington: American Public Health Association; 1985.
 40. Epi Info. *A World Processing, Database and Statistic Program for Epidemiology on Microcomputers*. 6.04 ed. Atlanta: Centers for Disease Control and Prevention; 1993.
 41. Kamat AS, Nair PM. Identification of *Listeria innocua* as a biological indicator for inactivation of *Listeria monocytogenes* by some meat processing treatment. *Lebens Wiss Technol*. 1996;29:714–720.
 42. Kheadr EE, Vachon JF, Paquin P, Fliss I. Effect of dynamic high pressure on microbiological, rheological and microstructural quality of Cheddar cheese. *Int Dairy J*. 2002;12:435–446.
 43. Perni S, Jordan SJ, Andrew PW, Shama G. Biofilm development by *Listeria innocua* in turbulent flow regimes. *Food Control*. 2006;17:875–883.
 44. Carvalho JDG, Viotto WH, Kuaye AY. The quality of Minas Frescal cheese produced by different technological processes. *Food Control*. 2007;18:262–267.
 45. Antwi M, Bernaerts K, Van Impe JF, Geeraerd AH. Modelling the combined effects of structured food model system and lactic acid on *Listeria innocua* and *Lactococcus lactis* growth in mono and coculture. *Int J Food Microbiol*. 2007;120:71–84.
 46. Bermúdez-Aguirre D, Barbosa-Cánovas GV. Study of butter fat content in milk on the inactivation of *Listeria innocua* ATCC 51742 by thermo-sonication. *Innov Food Sci Emerg Technol*. 2008;9:176–185.
 47. Noriega E, Laca A, Díaz M. Modelling of diffusion-limited growth for food safety in simulated cheeses. *Food Bioprod Process*. 2008;86:122–129.
 48. Rogga KJ, Samelis J, Kakouri A, Katsiari MC, Savvaidis IN, Kontominas MG. Survival of *Listeria monocytogenes* in Galotyri, a traditional Greek soft acid-curd cheese, stored aerobically at 4 °C and 12 °C. *Int Dairy J*. 2005;15:59–67.
 49. Gameiro N, Ferreira-Dias S, Ferreira M, Brito L. Evolution of *Listeria monocytogenes* populations during the ripening of naturally contaminated raw ewe's milk cheese. *Food Control*. 2007;18:1258–1262.
 50. Linton M, Mackle AB, Upadhyay VK, Kelly AL, Patterson MF. The fate of *Listeria monocytogenes* during the manufacture of Camembert-type cheese: a comparison between raw milk and milk treated with high hydrostatic pressure. *Innov Food Sci Emerg Technol*. 2008;9:423–428.
 51. Galinari E, Nóbrega JED, Andrade NJD, Ferreira CLDLF. Microbiological aspects of the biofilm on wooden utensils used to make a Brazilian artisanal cheese. *Braz J Microbiol*. 2014;45:713–720.
 52. Melo J, Andrew PW, Faleiro ML. *Listeria monocytogenes* in cheese and the dairy environment remains a food safety challenge: the role of stress responses. *Food Res Int*. 2015;67:75–90.
 53. Cokal Y, Dagdelen A, Cenet O, Gunsen U. Presence of *L. monocytogenes* and some bacterial pathogens in two Turkish traditional foods, Mihalic cheese and Hosmerim dessert. *Food Control*. 2012;26:337–340.
 54. Williams AG, Withers SE. Microbiological characterisation of artisanal farmhouse cheeses manufactured in Scotland. *Int J Dairy Technol*. 2010;63:356–369.
 55. De Buyser M-L, Dufour B, Maire M, Lafarge V. Implication of milk and milk products in food-borne diseases in France and in different industrialised countries. *Int J Food Microbiol*. 2001;67:1–17.
 56. Silbernagel KM, Carver CN, Jechorek RP, Johnson RL. Evaluation of VIDAS *Listeria monocytogenes* II (LMO2) immunoassay method for the detection of *Listeria monocytogenes* in Foods: collaborative study. *J AOAC Int*. 2004;87:903–918.
 57. Gangar V, Curiale MS, D'Onorio A, Schultz A, Johnson RL, Atrache V. VIDAS® enzyme-linked immunofluorescent assay for detection of *Listeria* in foods: collaborative study. *J AOAC Int*. 2000;83:903–918.
 58. Meyer C, Fredriksson-Ahomaa M, Sperner B, Martlbauer E. Detection of *Listeria monocytogenes* in pork and beef using the VIDAS® LMO2 automated enzyme linked immunoassay method. *Meat Sci*. 2011;88:594–596.

59. Sewell AM, Warburton DW, Boville A, Daley EF, Mullen K. The development of an efficient and rapid enzyme linked fluorescent assay method for the detection of *Listeria* spp. from foods. *Int J Food Microbiol.* 2003;81:123–129.
60. Aldus CF, Van Amerongen A, Ariens RM, Peck MW, Wichers JH, Wyatt GM. Principles of some novel rapid dipstick methods for detection and characterization of verotoxigenic *Escherichia coli*. *J Appl Microbiol.* 2003;95:380–389.
61. Health Canada. Annex 4.4: Procedure for the Statistical Evaluation and Calculation of Performance Parameters of a New Alternative Qualitative Method Compared to a Reference Cultural Method; 2011. Accessed 04.07.11.
62. Borelli BM, Ferreira EG, Lacerda ICA, et al. Enterotoxigenic *Staphylococcus* spp. and other microbial contaminants during production of Canastra cheese, Brazil. *Braz J Microbiol.* 2006;37:545–550.
63. Cardoso VM, Dias RS, Soares BM, Clementino LA, Araújo CP, Rosa CA. The influence of ripening period length and season on the microbiological parameters of a traditional Brazilian cheese. *Braz J Microbiol.* 2013;44:743–749.
64. Imran M, Bre JM, Gueguen M, Vernoux JP, Desmasures N. Reduced growth of *Listeria monocytogenes* in two model cheese microcosms is not associated with individual microbial strains. *Food Microbiol.* 2013;33:30–39.
65. Perin LM, Moraes PM, Viçosa GN, Silva Júnior A, Nero LA. Identification of bacteriocinogenic *Lactococcus* isolates from raw milk and cheese capable of producing nisin A and nisin Z. *Int Dairy J.* 2012;25:46–51.
66. Souza CFV, Dalla Rosa T, Ayu MAS. Changes in the microbiological and physicochemical characteristics of Serrano cheese during manufacture and ripening. *Braz J Microbiol.* 2003;34:260–266.