International Journal of Food Microbiology 173 (2014) 21–29

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Pulsed-Field Gel Electrophoresis characterization of *Listeria monocytogenes* isolates from cheese manufacturing plants in São Paulo, Brazil

Giovana V. Barancelli [a], Tarsila M. Camargo [b], Natália G. Gagliardi [a], Ernani Porto [b], Roberto A. Souza [c], Fabio Campioni [c], Juliana P. Falcão [c], Ernesto Hofer [d], Adriano G. Cruz [e], Carlos A.F. Oliveira [a,*]

[a] Departamento de Engenharia de Alimentos, Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Av. Duque de Caxias – Norte, 225, CEP 13635–900, Pirassununga, SP, Brazil
[b] Departamento de Agroindústria, Alimentos e Nutrição, Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, Piracicaba, SP, Brazil
[c] Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil
[d] Laboratório de Zoonoses Bacterianas, Instituto Oswaldo Cruz, Rio de Janeiro, RJ, Brazil
[e] Instituto Federal de Educação, Ciência e Tecnologia do Rio de Janeiro, Rio de Janeiro, RJ, Brazil

ARTICLE INFO

Article history:
Received 22 July 2013
Received in revised form 9 December 2013
Accepted 21 December 2013
Available online 28 December 2013

Keywords:
L. monocytogenes
Cheese
Environment
PFGE

ABSTRACT

This study aimed to evaluate the occurrence of *Listeria monocytogenes* in cheese and in the environment of three small-scale dairy plants (A, B, C) located in the Northern region state of São Paulo, Brazil, and to characterize the isolates using conventional serotyping and PFGE. A total of 393 samples were collected and analyzed from October 2008 to September 2009. From these, 136 came from dairy plant A, where only *L. seeligeri* was isolated. In dairy plant B, 136 samples were analyzed, and *L. innocua, L. seeligeri* and *L. welshimeri* were isolated together with *L. monocytogenes*. In dairy plant C, 121 samples were analyzed, and *L. monocytogenes* and *L. innocua* were isolated. Cheese from dairy plants B and C were contaminated with *Listeria* spp, with *L. innocua* being found in Minas frescal cheese from both dairy plants, and *L. innocua* and *L. monocytogenes* in Prato cheese from dairy plant C. A total of 85 *L. monocytogenes* isolates were classified in 3 serotypes: 1/2b, 1/2c, and 4b, with predominance of serotype 4b in both dairy plants. The 85 isolates found in the dairy plants were characterized by genomic macrorestriction using *Apa*I and *Asc*I with Pulsed Field Gel Electrophoresis (PFGE). Macrorestriction yielded 30 different pulsotypes. The presence of indistinguishable profiles repeatedly isolated during a 12-month period indicated the persistence of *L. monocytogenes* in dairy plants B and C, which were more than 100 km away from each other. Brine used in dairy plant C contained more than one *L. monocytogenes* lineage. The routes of contamination were identified in plants B and C, and highlighted the importance of using molecular techniques and serotyping to track *L. monocytogenes* sources of contamination, distribution, and routes of contamination in dairy plants, and to develop improved control strategies for *L. monocytogenes* in dairy plants and dairy products.

1. Introduction

Listeria monocytogenes is an important human pathogen that occurs in several food processing environments, including dairy processing plants (Kathariou, 2002). Listeriosis has been recognized as a serious public health hazard, with high mortality rates in susceptible individuals, such as the elderly and immunocompromised (Swaminathan and Gerner-Smidt, 2007). Consumption of contaminated dairy products has been associated with cases and outbreaks of human listeriosis (Leite et al. 2006). In Brazil, human listeriosis is underdiagnosed and underreported (Silva et al., 2010), and there is no report on foodborne cases (Brito et al., 2008), although *L. monocytogenes* is frequently isolated from dairy products (Zaffari et al., 2007; Brito et al., 2008; Abrahão et al., 2008; De Nes et al., 2010; Barancelli et al., 2011).

The main types of Brazilian commercial cheeses include Minas Frescal (non-ripened, fresh cheese) and Prato (ripened cheese) varieties, accounting for nearly 30% of the 640,000 kg of cheese produced annually in the country. However, only Minas Frescal cheese was positive for *L. monocytogenes* in previous reports (Carvalho et al., 2007; Brito et al., 2008).

The ability of *L. monocytogenes* to survive long periods in adverse conditions, and to colonize and persist in food processing environments is a threat to the food industry, especially the dairy industry (Miettinen et al., 1999; Kathariou, 2002; Wagner et al., 2006). Pulsed Field Gel Electrophoresis (PFGE) has been successfully used in *L. monocytogenes* typing in epidemiological surveys (Miettinen et al., 1999; Barret et al., 2006; Neves et al., 2008; Latorre et al., 2009; Sauders et al., 2009), and the association with serotyping is

* Corresponding author. Tel.: +55 19 3565 4173; fax: +55 19 3565 4284.
E-mail address: carlosaf@usp.br (C.A.F. Oliveira).

0168-1605/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijfoodmicro.2013.12.018

widely recommended in epidemiological studies to assess the persistence of this pathogen in the food processing environment (Chasseignaux et al., 2001).

In Brazil, there is little information about genotypic variation in *L. monocytogenes* isolated from the environment of dairy industries, especially in small-scale cheese processing plants that produce Minas Frescal and Prato cheeses. Furthermore, there are no studies in Brazil comparing the genetic profile of *L. monocytogenes* isolated from different dairy plants. In this context, this study aimed to isolate and characterize *L. monocytogenes* from cheeses and from the environment of three small-scale cheese processing plants located in the Northeastern region of São Paulo State, Brazil, using serotyping and PFGE.

2. Material and methods

2.1. Sampling procedure

The three dairy plants, located in the northeastern region of the state of São Paulo, were not connected, and were located 100 km away from each other. They did not have raw milk suppliers in common. The volume of cow's milk processed per day and the number of farms that supplied milk to the dairy plants were: 7,000 liters and 60 farms (dairy plant A); 12,000 liters and 60 farms (dairy plant B); and 3,000 liters and 55 farms (dairy plant C). Cheese processing was similar in the three plants using pasteurized milk, without the use of lactic acid bacteria in the procedure.

The three plants used the same type of mold for the cheese — round, plastic, and with drainage holes in the bottom. After some minutes in the mold, the cheese was turned over inside of it. Cheese in molds was placed in plastic crates with drainage holes, which were kept in cooling chambers (4 to 7 °C) for 16 h. Afterwards, the molds were removed and the cheese was placed in plastic bags that were closed with metal clamps. It was then kept in the cooling chamber (4 to 7 °C) inside plastic crates, until the moment of sale. None of the plants performed routine monitoring of *Listeria* spp. in the plant environment or in the cheese. Plant A had the best level of Good Manufacturing Practices (GMP), as it complied with more than 76% of the items in the GMP evaluation cheklist adopted by the Brazilian Inspection Authority (Brazil, 2002). Plants B and C, however, were categorized as an intermediate GMP level, since they complied with 51–75% of the GMP items.

From October 2008 to September 2009, samples were collected in the three cheese plants (4 visits each, 2-monthy intervals, totaling 12 visits). During each sampling procedure, the following samples were collected in sterilized containers: 1000 mL of water (with 1.0 mL of 10% sodium thiosulfate solution to each 100 mL), 500 mL of raw and pasteurized milk, brine (from chilled brine tanks), 500 g of semi-finished cheese (in molds $n = 5$), and packaged Minas Frescal cheese ($n = 5$) from one batch. Samples from food contact utensils [raw milk filter, vats, spatulas, molds ($n = 15$), tables, worker gloves ($n = 1$ to 3), plastic crates ($n = 5$)], and of non-food contact surfaces (floor, drains, walls, and platforms) were also collected. After the third sampling from each batch, together with samples of cheese from the molds and packed cheese, three samples (each of 15 combined cheese samples) from the lateral and upper surfaces of Minas Frescal cheese were collected with sterile gloves and sponges (Inlab Diagnostica) moistened with saline solution (0.85%) immediately before packaging.

At the time of the fourth sampling, samples of semi-finished Minas Frescal cheese were not available in dairy plant C. Therefore, just before packaging, samples were collected from Prato cheese surfaces. Samples from food contact and non-food contact surfaces were collected with sponges or cotton swabs (Inlab Diagnostica) moistened with saline solution (0.85%) containing peptone (0.1%) and neutralizers for the following chemical disinfectants: sodium thiosulfate (0.01%); polysorbate (0.5%), and soy lecithin (0.07%) (Evancho et al., 2002). Flat surfaces, such as tables, vats, walls, and floors were sampled with sterile gloves and a sponge over an 1.00 m^2 area, using sterilized templates for guidance.

The inside and outside of each cheese mold and plastic crate were sampled with cotton swabs and a sponge over a 27-cm^2 and 1000-cm^2 area, respectively. Platforms were sampled with cotton swabs for the cracks on the surface to be reached over a total area of 400 cm^2. Spatulas and curd-cutting utensils were sampled all over the surface that was in contact with the curd. For drained samples, all accessible surfaces were wiped with a sponge, including sidewalls and grates. The packaging equipment and raw milk filter were sampled with a sponge. Worker gloves were sampled with cotton swabs.

After sampling, sponges were put into bags (Inlab Diagnostica) containing 60 mL of Buffered *Listeria* Enrichment Broth Base (BLEB; Difco), whereas cotton swabs were placed in tubes containing 15 mL of the same broth. All samples were refrigerated immediately after collection and shipped to the laboratory in coolers with ice (4–8 °C), and analyzed on the same day.

2.2. Isolation of Listeria species

Analysis of *L. monocytogenes* was carried out according to the U.S. Food and Drug Administration method (Hitchins, 2003). *L. monocytogenes* ATCC 7644 was used as a positive control. Briefly, 25 g or mL of product samples were added to 225 mL of BLEB. From the second sampling of each plant onwards, 50 g or mL of the samples were added to 450 mL of BLEB to increase the possibility of isolation. Filtration (Silva et al., 2005) with a 0.45-μm pore membrane was used to analyze the 1,000-mL water samples. After filtration, the membrane was placed in 100 mL of BLEB. Samples collected with sponges and cotton swabs were vortexed and homogenized in a stomacher. Solid samples with BLEB were also homogenized in the stomacher for 60 s and incubated at 30 °C for 48 h. After 4 h of incubation, acriflavine (10 mg/L), nalidixic acid (40 mg/L), and cycloheximide (50 mg/L) were added to the mixture. One loopful of each BLEB mixture was streaked onto *Listeria* agar plates, according to Ottaviani and Agosti (ALOA; AES Chemunex), and onto Oxford agar (Oxoid) plates. The ALOA and Oxford agar plates were incubated at 37 °C/24 h and 35 °C/24–48 h, respectively. From each positive sample, three typical colonies of *Listeria* from Oxford agar, and of *L. monocytogenes* and/or *Listeria* from ALOA were transferred to Tryptone Soy Agar plus 0.6% Yeast Extract (TSA + YE; Difco) plates, and incubated at 37 °C for 24 h. Isolates that were Gram-positive rods and catalase positive, with typical umbrella-like motility in semisolid motility medium (SIM; Difco) after incubation at 25 °C for 7 days, were considered to be *Listeria.*

L. monocytogenes was differentiated from other species of *Listeria* by using API *Listeria* (BioMeriéux) kit. The final confirmation was provided by Christie–Atkins–Munch–Peterson (CAMP) Test (Hitchins, 2003). Additionally, *Listeria* found in each positive sample was enumerated by using the Most Probable Number (MPN) with serial dilutions in BLEB, following the procedures described by Blodgget (1998).

2.3. Serotyping of Listeria monocytogenes

All isolates identified as *L. monocytogenes* were serotyped in the *Laboratório de Zoonoses Bacterianas* of Instituto Oswaldo Cruz/RJ, according to Seeliger and Höhne (Seeliger and Höhne, 1979).

2.4. PFGE analysis of Listeria monocytogenes

All *L. monocytogenes* isolates were submitted to chromosomal DNA and restriction endonuclease digestion strictly following the procedures described by the U.S. Centers for Disease Control (Pulsenet International, 2009). PFGE analysis was carried out in a CHEF-DR III system (Bio-Rad, Hercules, CA). Running parameters were as follows: 6 V/cm; angle: 120°; temperature: 14 °C; initial switch: 4 s; final switch: 40s; length: 19 h. After the electrophoresis run was completed, the gel was stained with 50 μL of ethidium bromide (Sigma-Aldrich, Saint Louis, MO) solution (10 mg/mL) in 500 mL of distilled water for 20 min

in a covered container, and de-stained in 500 mL of fresh distilled water for 30 min. Gel DocXR (Bio-Rad, Hercules, CA) was used to capture the images under UV light. A molecular weight marker (Lambda Ladder PFG Marker, BioLabs, São Paulo, Brazil) with an effective range from 48.5 kb to 727.5 kb, was used. Bands above or below this range were not included in the analysis.

The images were analyzed using BioNumerics software, v.5.1 (Applied Maths). Banding patterns were marked using a number of different stained gel images, in addition to the curves provided by Bionumerics. Similarities dendrograms were constructed by the unweighted pair group method using arithmetic means (UPMGA), and Dice coefficients were used to calculate similarities between profiles with tolerance and optimization positions of 1.2% for the enzymes *Asc*I and *Apa*I (Martin et al., 2006). Besides *L. monocytogenes* isolated from the dairy plants (N = 85), results of PFGE of *L. monocytogenes* ATCC 7644 were included to build the dendogram. Isolates that showed 100% similarity after DNA digestion with *Apa*I and *Asc*I were considered as belonging to the same PFGE patterns (indistinguishable), and any difference between two profiles was considered sufficient to report patterns as different (Barret et al., 2006).

3. Results

3.1. Occurrence of Listeria species

The number of samples contaminated with *Listeria* spp. species in each sampling site is presented in Table 1. None of the samples of water, raw milk, pasteurized milk or curd used in the manufacture of Minas Frescal cheese were positive for *Listeria* spp. Among samples that tested positive in any of the dairy factories, the percentage of contamination for *L. monocytogenes* was 7.1% (28/393). In dairy plant A, only the species *L. seeligeri* was isolated in 4 of 136 samples (2.9%), and it came from the cooling chamber. In dairy plant B, besides *L. monocytogenes* isolated in 17 of 136 samples (12.5%), the following species were also isolated: *L. innocua* (19 of 136; 14.0%), *L. seeligeri* (4 of 136; 2.9%), and *L. welshimeri* (1 of 136; 0.7%). Dairy plant C showed environmental samples, brine and cheeses positive for *L. monocytogenes* in 11 of 121 samples (9.1%) and *L. innocua* (29 of 121; 24.0%). Minas Frescal cheese in dairy plant C showed two positive samples with *L. innocua* populations lower than 0.3 MPN per gram, and one sample showed 9.2 MPN per gram. With respect to the samples of Minas frescal cheese from dairy factory B, and Prato cheese from dairy factory C (Table 1), it was not possible to determine the *Listeria* population, because sample collection was carried out with a sponge without any limiting of the sampling area on the surface of cheeses.

3.2. Conventional serotyping of Listeria monocytogenes

Antigenic characterization of the 85 isolates identified as *L. monocytogenes* showed three serotypes: 4b, 1/2b, and 1/2c. The percentage values of these serotypes in each plant are presented in Table 2. The distribution of these serotypes in the respective isolation sites during sample collection are shown in Table 3.

3.3. Molecular characterization of Listeria monocytogenes

The enzymes *Apa*I and *Asc*I made possible the typing of the 85 isolates confirmed as *L. monocytogenes*. The sites of isolation of each pulsotype in each dairy plant and the order of sample collection are summarized in Table 3. Figs. 1 and 2 show dendrograms of the isolates based on *Apa*I and *Asc*I digestion, respectively. The analysis with *Apa*I and *Asc*I differentiated the 85 isolates plus *Listeria monocytogenes*

Table 1
Occurrence of *Listeria* spp. isolates in cheese manufacturing plants from São Paulo, Brazil.

Sampling site	N	No. of positive samples			
		L. monocytogenes	*L. seeligeri*	*L. innocua*	*L. welshimeri*
Plant A					
Water	4	0	0	0	0
Raw milk	8	0	0	0	0
Pasteurized milk	4	0	0	0	0
Curd (Minas frescal)	4	0	0	0	0
Minas Frescal cheese[a]	46	0	0	0	0
Non-food contact surfaces[b]	29	0	4	0	0
Food contact surfaces[c]	41	0	0	0	0
Plant B					
Water	4	0	0	0	0
Raw milk	8	0	0	0	0
Pasteurized milk	4	0	0	0	0
Curd (Minas frescal)	4	0	0	0	0
Minas Frescal cheese[a]	46	0	0	1	0
Non-food contact surfaces[b]	31	16	4	17	1
Food contact surfaces[c]	39	1	0	1	0
Plant C					
Water	4	0	0	0	0
Raw milk	4	0	0	0	0
Pasteurized milk	4	0	0	0	0
Curd (Minas frescal)	3	0	0	0	0
Minas Frescal cheese[d]	38	0	0	3	0
Prato cheese (surface) [e]	3	2	0	3	0
Brine	4	2	0	3	0
Non-food contact surfaces[b]	23	5	0	18	0
Food contact surfaces[c]	38	2	0	2	0
Total	393	28	8	48	1

N: Number of samples analyzed.
[a] Sampling of Minas Frescal cheese comprised 20 samples of cheese in molds, 20 samples of packaged cheese and 6 samples collected from cheese surfaces before packaging.
[b] Surfaces of floor, drains, walls, and platforms.
[c] Surfaces of raw milk filter, vats, spatulas, cheese curd cutting utensils, molds, tables, worker gloves, plastic crates, and packaging equipment.
[d] Sampling of Minas Frescal cheese comprised 15 samples of cheese in molds, 20 samples of packaged cheese, and 3 samples collected from cheese surfaces before packaging.
[e] Each sample was collected from surfaces of 15 units of cheese immediately before packaging.

Table 2
Occurrence of *Listeria monocytogenes* serotypes in cheese manufacturing plants from São Paulo, Brazil.[a]

	Plant B	Plant C	Total
Serotype	*n* (%)	*n* (%)	*n* (%)
4b	45 (82)	26 (86)	71 (84)
1/2b	10 (18)	2 (7)	12 (14)
1/2c	0 (0)	2 (7)	2 (2)
Total	55 (100)	30 (100)	85 (100)

n: Number of isolates.
[a] Results reported for plants B and C, as *Listeria monocytogenes* was not isolated in plant A.

ATCC 7644 in 23 and 19 PFGE profiles, respectively, named Ap1–Ap23 for profiles distinguished by *Apa*I and As1–As19 for profiles distinguished by *Asc*I, dividing the isolates into 31 different pulsotypes (listed from I to XXXI) (Table 4).

The pulsotypes were clustered in a similar manner with *Apa*I and *Asc*I, except for VI, VII and XVI, as shown in Figs. 1 and 2. All the PFGE profiles were serotype specific, with the exception of one isolate (34B) digested by *Asc*I, hence, identical PFGE types were not found among different serotypes with *Apa*I and *Asc*I. Isolates 9C and 15C (pulsotype XVI), and 34B (pulsotype XXIV) were from serotype 1/2b, and were at the same branch of others from serotype 4b digested by *Apa*I (Fig. 1). However, when *Asc*I was used, the isolate 34B showed an indistinguishable profile from the others isolates from serotype 4b (Fig. 2). All the isolates from dairy plant B and 93% of isolates from plant C belong to lineage I of *L. monocytogenes* (Ragon et al., 2008), represented by serotypes 4b and 1/2b (Figs. 1 and 2). The pulsotypes that were less related to the others, with less than 65% similarity, were XXX and XXI, which included isolates from brine in dairy plant C, and *L. monocytogenes* ATCC 7644 from serotype 1/2c, which belongs to lineage II of *L. monocytogenes* (Ragon et al., 2008).

4. Discussion

Dairy plants B and C, in which cleaning conditions were inferior to those in dairy plant A, showed the greatest incidence of *Listeria* spp. *L. monocytogenes* was isolated in all samplings from these dairy plants, whereas the only species isolated in dairy plant A was *L. seeligeri*. Isolation of *Listeria* spp. is a relevant finding, as *L. monocytogenes* may be found in the same sites where other *Listeria* species are isolated (Kells and Gilmour, 2004).

In Brazil, previous studies showed that the occurrence of *L. monocytogenes* in the environment of dairy plants range from zero to 43% (Silva et al., 2003; Borges, 2006; Brito et al., 2008). In other countries, wide variations in the incidence of *L. monocytogenes* have also been reported (0 to 90.5%) in dairy plant environments (Wagner et al., 2006; D'Amico and Donnelly, 2009). Kabuki et al. (2004) analyzed the environment and cheese samples of three plants that manufactured Latino fresh cheese in New York, and found *L. monocytogenes* in 27 of 246 samples of the environment, with positive results for *L. monocytogenes* in the final product of one plant. Similar results were found in the present study, in which *L. monocytogenes* was isolated in cheeses only from dairy plant C.

The isolation of *L. monocytogenes* from plastic crates in dairy plants B and C shows the importance of these utensils as sources and vehicles for the dissemination of this bacterium in dairy plants, as already pointed out by Kabuki et al. (2004). The absence of *L. monocytogenes* in samples of raw milk confirms the low prevalence or even the absence of the pathogen in raw milk from Brazilian herds, according to previous studies that also did not isolate the microorganism (Destro et al., 1991; Casarotti et al., 1994; Arcuri et al., 2006; Nero et al., 2008). The absence of *L. monocytogenes* in the samples of pasteurized milk also confirms that the product is not a common source of this bacterium, as reported by Destro et al. (1991) and Frye and Donnelly (2005).

The occurrence of *L. monocytogenes* in cheese is a cause for concern because these products are often consumed without any further processing. Other important factors that should be considered concerning the isolation of *L. monocytogenes* in cheese are the shelf life (180 days) and storage period of the product under refrigeration. Low temperatures allow *Listeria* multiplication, and may lead to infectious doses in the products, and/or cross-contamination in the food preparation environment. The isolation of *L. monocytogenes* from the brine in dairy plant C indicates the possible transmission to the cheese, as occurred in Prato cheeses in that dairy plant. *L. monocytogenes* tolerates large concentrations of NaCl and pH 4.9 (Larson et al., 1999). It is,

Table 3
Occurrence of *Listeria monocytogenes* in cheese manufacturing plants B and C, and results of serotyping and PFGE typing with *Apa*I and *Asc*I.

	Sampling order [a]							
	1		2		3		4	
Site of isolation	Serotype	Pulsotype	Serotype	Pulsotype	Serotype	Pulsotype	Serotype	Pulsotype
Dairy plant B								
Floor (pasteurization room)	4b(1)[b] 1/2b(1)	X IV	4b(2)	XV	–		1/2b(4)	I
Drain in cooling chamber	4b(1)	XVII	4b(6)	XVII, XVIII	4b(5)	XVIII	4b(3) 1/2b(1)	XVIII, XXIII XXIV
Platform (cooling chamber1)	–	–	–	–	4b(2)	XVIII, XXII	4b(3)	XVIII, XIX
Floor (cooling chamber 1)	–	–	4b(4)	XVII, XVIII	4b(3) 1/2b(1)	XVIII, XXII II	4b(4)	XVII, XVIII
Floor (cooling chamber 2)	4b(1)	XX	4b(2) 1/2b(1)	XIV, XVIII V	1/2b(2)	II, III	4b(4)	VIII, IX
Plastic crates	–	–	–	–	–	–	4b(4)	XVIII
Dairy plant C								
Platform (cooling chamber)	–	–	4b(1)	XXI	–	–	4b(1)	XXIX
Floor (cooling chamber)	4b(2)	XXV	–	–	4b(2) 1/2b(1)	XI, XII XVI	4b(5)	XIV, XXVII, XXVIII
Plastic crates	–	–	–	–	–	–	4b(3) 1/2b(1)	VI, VII, XIII XVI
Worker (gloves)	–	–	4b(3)	XXI	–	–	–	–
Brine	–	–	–	–	1/2c(2)	XXX	4b(3)	XXVI
Prato cheese	NC	NC	NC	NC	NC	NC	4b(6)	XVII, XVIII

NC: sample not collected.
[a] Sampling dates at plant B were: 1. Nov/2008, 2. Feb/2009, 3. May/2009, and 4. Aug/2009; For plant C, sampling dates were: 1. Dec/2008, 2. Mar/2009, 3. Jun/2009, and 4. Sep/2009.
[b] Values in parenthesis are numbers of isolates of the serotype in each sampling site.

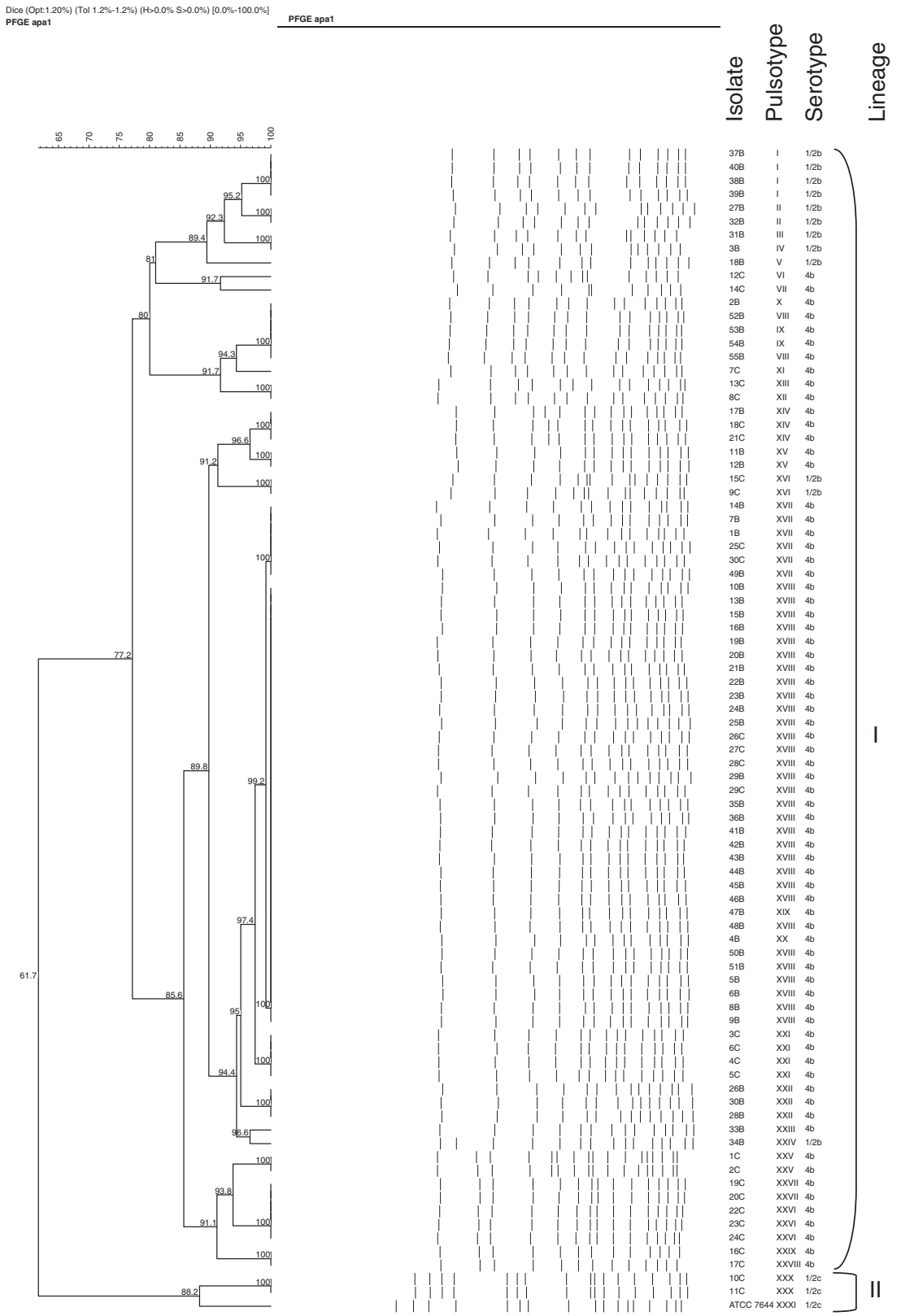

Fig. 1. Dendrogram obtained with *Apa*I demonstrating the genetic relationships of the 85 isolates collected in plants B and C and *Listeria monocytogenes* ATCC 7644.

therefore, able to survive in commercial brines (Wagner et al., 2006; Barancelli et al., 2011).

In dairy plants in different regions of the world, the prevalence of *Listeria* spp. varies. D'amico and Donnelly (2009) showed low prevalence (10.6%) of *Listeria* spp. in the environment of small artisan-like dairy factories that manufacture cheese with raw milk in the USA, and *L. monocytogenes* was isolated from the environment of 2 from 8 plants analyzed. Wagner et al. (2006) isolated *L. monocytogenes* from

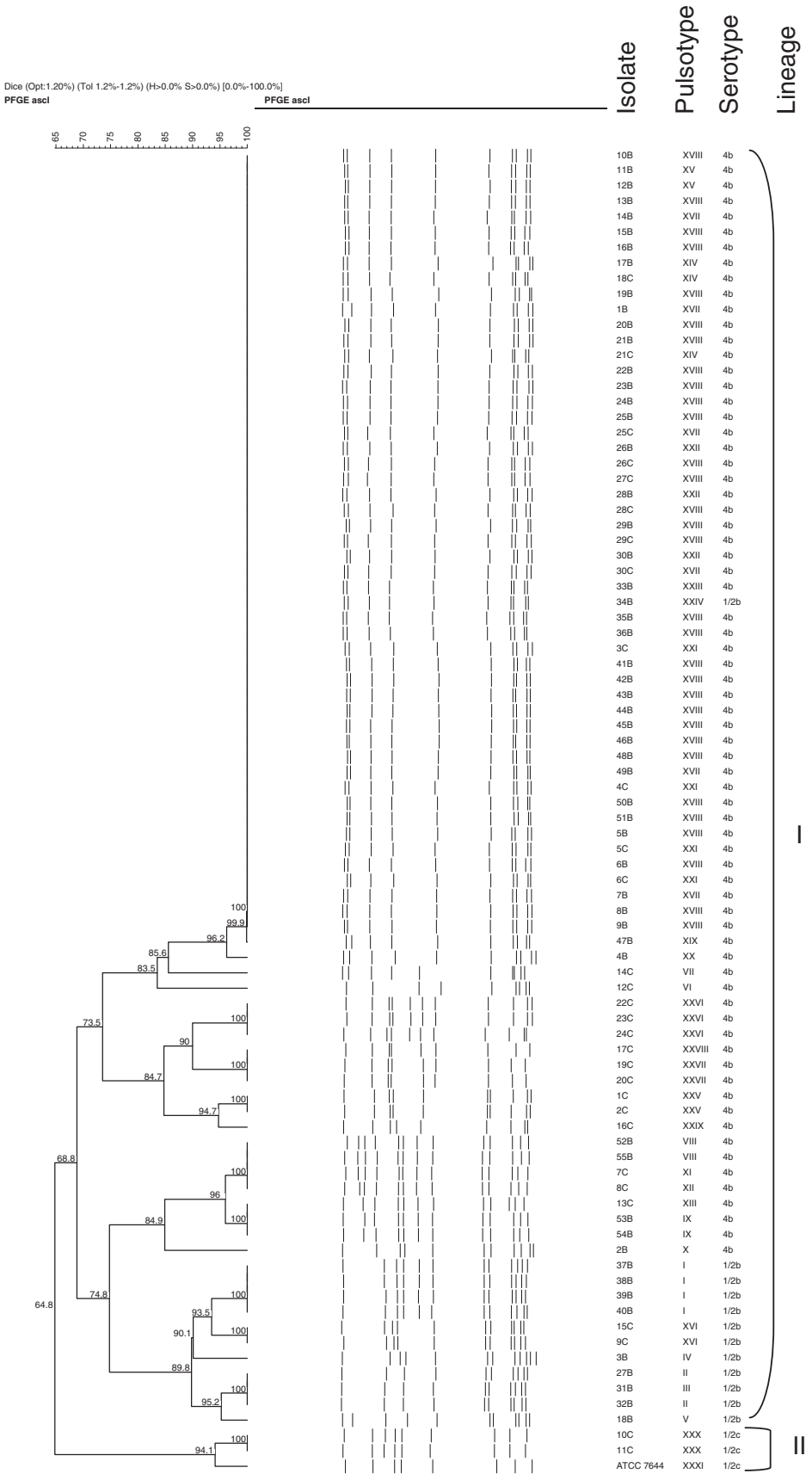

Fig. 2. Dendrogram obtained with *Asc*I demonstrating the genetic relationships of the 85 isolates obtained in plants B and C and *Listeria monocytogenes* ATCC 7644.

Table 4
Pulsotypes of *L. monocytogenes* isolates from plants B and C, according to the combined typing with *Apa*I and *Asc*I restriction patterns.

Isolate code [a]	Restriction pattern		Pulsotype
	*Apa*I	*Asc*I	
37B, 38B, 39B, 40B	Ap1	As13	I
27B, 32B	Ap2	As16	II
31B	Ap3	As16	III
3B	Ap3	As15	IV
18B	Ap4	As17	V
12C	Ap5	As5	VI
14C	Ap6	As4	VII
52B, 55B	Ap7	As10	VIII
53B, 54B	Ap7	As11	IX
2B	Ap7	As12	X
7C	Ap8	As10	XI
8C	Ap9	As10	XII
13C	AP9	As11	XIII
17B, 18C, 21C	Ap10	As1	XIV
11B, 12B	Ap11	As1	XV
9C, 15C	Ap12	As14	XVI
1B, 7B, 14B, 49B, 25C, 30C	Ap13	As1	XVII
5B, 6B, 8B, 9B, 10B, 13B, 15B, 16B, 19B, 20B, 21B, 22B, 23B, 24B, 25B, 29B, 35B, 36B, 41B, 42B, 43B, 44B, 45B, 46B, 48B, 50B, 51B, 26C, 27C, 28C, 29C	Ap14	As1	XVIII
47B	Ap14	As2	XIX
4B	Ap14	As3	XX
3C, 4C, 5C, 6C	Ap15	As1	XXI
26B, 28B, 30B	Ap16	As1	XXII
33B	Ap17	As1	XXIII
34B	Ap18	As1	XXIV
1C, 2C	Ap19	As8	XXV
22C, 23C, 24C	Ap20	As6	XXVI
19C, 20C	Ap20	As7	XXVII
17C	Ap21	As7	XXVIII
16C	Ap21	As9	XXIX
10C, 11C	Ap22	As18	XXX

[a] Letters B and C in each isolate code indicate the dairy plants from which they were isolated.

50 (27.6%) small processing plants in Austria. In Brazil, Silva et al. (2003) isolated *Listeria* spp. from two dairy plants in Bahia, and found *L. monocytogenes* in only one of them.

The predominance of serotype 4b in dairy plants B and C is of great public health importance, because this serotype is frequently involved in outbreaks of human listeriosis (Graves et al., 2007; Swaminathan and Gerner-Smidt, 2007). Therefore, it is likely that serotype designation is associated with virulence potential. This was an unexpected finding, because this serotype is not the most frequent one in food and food-processing environments (Kathariou, 2002). In Brazil, serotype 1/2a has been the most frequently one isolated from dairy products (Hofer et al., 2006; Brito et al., 2008; Abrahão et al., 2008). The predominance of serotype 4b in the two dairy factories may suggest its endemic nature, which may be associated with peculiarities of these plants. Plants B and C had similar characteristics, such as production on a small scale, deficient cleaning processes, and lack of training of food handlers, as well as non-compliance with Good Manufacturing Practices during cheese processing. Similar to the results observed in the present study, previous reports showed the predominance of serotype 4b in samples of small dairy plants and cheese in Portugal (Pintado et al., 2005; Leite et al., 2006; Chambel et al., 2007), and high prevalence in dairy industries in Austria (Wagner et al., 2006). In Algiers, Hamdi et al. (2007) reported 100% of prevalence of serotype 4b in raw milk, while Aurora et al. (2009) found 72% of prevalence for this serotype in raw milk and milk products in India.

In the PFGE analysis, the use of two enzymes (*Apa*I and *Asc*I) showed greater discriminatory power than when a single enzyme was used, reinforcing the importance of the combination of the two enzymes in the analysis of *L. monocytogenes*, as recommended by Fugett et al. (2007) and Neves et al. (2008).

There were three pulsotypes (XIV, XVII and XVIII) that were shared by dairy plants B and C (Table 3). In this group, isolates from Prato cheese (pulsotypes XVII and XVIII) in dairy factory C showed PFGE profiles indistinguishable from isolates of dairy plant B found in drains, plastic crates, platforms, and the floor of the cooling chambers. Pulsotype XIV was found on the cooling chamber floors of both plants. The presence of isolates of the same pulsotype in both dairy factories suggests that these are *L. monocytogenes* subtypes that come from a common source or are widely spread in nature, and may have caused the contamination of the plants. The existence of a single source of contamination for dairy plants B and C does not seem likely, since the plants were not connected, and did not have suppliers of raw milk in common. The hypothesis of wide distribution of these pulsotypes is more plausible. Studies in Austria (Wagner et al., 2006) and Portugal (Leite et al., 2006) showed similar results, with the occurrence of the same PFGE profile in epidemiologically unrelated dairy plants, suggesting that some subtypes of *L. monocytogenes* are widely distributed and become endemic because they become adapted to specific ecological niches, such as the cheese production environment.

Pulsotypes that clustered with both enzymes can be considered presumably persistent in the plants, as showed in Table 3. These results indicate the ability of the lineages to adapt to niches and biofilms, and/or resistance to cleaning procedures, as demonstrated by previous studies (Miettinen et al., 1999; Autio et al., 2003; Sauders et al., 2009). Lineages that persist in food industries or cause outbreaks may present genetic alterations that result in PFGE patterns with differences of up to three bands (Graves et al., 2005). Therefore, Sauders et al. (2009) considered that isolates presenting up to three bands of difference in *Apa*I + *Asc*I profiles belonged to the same PFGE profile, and were considered persistent in the analysis of the diversity and persistence of *L. monocytogenes* in food retail stores. The diversity of PFGE profiles observed in dairy plants B and C is different from the findings of the only study carried out in Brazil, in which *L. monocytogenes* was analyzed in a dairy plant and all the 344 isolates belonged to serotype 1/2a, with undistinguishable profiles (*Apa*I and *Asc*I), demonstrating a homogenous contamination pattern in the facilities (Brito et al., 2008).

Besides the persistence of the lineages, PFGE highlighted important routes of contamination by *L. monocytogenes*. Therefore, in dairy factory B, in the fourth sampling, the same pulsotype was isolated (XVIII) from the floor, drain, platform in cooling chamber 1, and plastic crates. In dairy factory C, highly similar pulsotypes were found in brine (XXVI) and on the floor of the cooling chamber (XXVII and XXVIII), with 90% and 91,1% similarity with *Asc*I and *Apa*I, respectively (Figs. 1 and 2). This fact suggests a route of contamination, and confirms the use of inadequate handling practices, such as placing the crates that were in direct contact with the floor inside the brine tank, causing contamination of the brine. Also in dairy plant C, the same pulsotype (XXI) was found on the gloves of a worker and on the platform of the cooling chambers in the second sampling, showing a new route of contamination coming from the floor. No common pulsotypes were found among the isolates in Prato cheese of dairy plant C (XVII, XVIII) and other sites of this plant in the same sampling, including the brine, but only similar pulsotypes in worker's gloves and the platform in the second sampling (pulsotype XXI) and on the floor of the cooling chamber (pulsotype XIV) in 4th sampling. The pulsotype XXX, isolated from the brine in plant C, was clustered with *Listeria monocytogenes* ATCC 7644 (serotype 1/2c, pulsotype XXXI), which is of human origin. According to Ragon et al. (2008), serotype 4b evolved from 1/2b, which could explain the isolate 34B from 1/2b serotype showing the same *Asc*I profile of others from serotype 4b (Fig. 2); as well isolates 34B, 9C and 15C, have been clustered with others isolates from serotype 4b with *Apa*I (Fig. 1). Discrepancies between PFGE profiles and serotypes have been reported previously. Margolles et al. (1998) found indistinguishable PFGE profiles among *L. monocytogenes* of different serotypes isolated from cheeses. Chasseignaux et al. (2001)

also observed clustering of *L. monocytogenes* isolates from different serotypes in the same pulsotype collected in poultry and pork processing plants.

In conclusion, the isolation of *L. monocytogenes* from different sites of dairy plants B and C, in all samplings, demonstrated that these facilities are sources of contamination of dairy products, and cleaning procedures for the control of this pathogen are necessary, including for the brine tank. The predominance of serotype 4b in both plants is a great public health concern due to its epidemiological importance. PFGE analysis demonstrated different sources of contamination and the persistence of lineages, making it difficult to control the pathogen in dairy plants B and C. The presence of the same *L. monocytogenes* pulsotype in the two plants suggests the wide distribution of the pathogen in nature and its adaptation to the dairy plant environment.

This study highlights the importance of using molecular techniques and serotyping to track *L. monocytogenes* sources of contamination, distribution, and routes of contamination in dairy plants, especially when rare profiles are indistinguishable and spread in the factory, in order to plan preventive measures to control this microbiological hazard in the industrial environment as well as in the final products.

Acknowledgements

The authors are grateful to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for the financial support.

References

Abrahão, W.M., Monteiro, C.L.B., Pontarolo, R., 2008. Occurrence of *Listeria monocytogenes* in cheese and ice cream produced in the State of Paraná, Brazil. Rev. Bras. de Ciências Farm. 44, 289–296.

Arcuri, E.F., Brito, V.P., Brito, J.R.F., Pinto, S.M., Angelo, F.F., Souza, G.N., 2006. Qualidade microbiológica do leite refrigerado nas fazendas. Arq. Bras. Med. Vet. Zootec. 58, 440–446.

Aurora, R., Prakash, A., Prakash, S., 2009. Genotypic characterization of *Listeria monocytogenes* isolated from milk and ready-to eat indigenous milk producs. Food Control 20, 835–839.

Autio, T., Keto-Timonen, R., Lundén, J., Björkroth, J., Korkeala, H., 2003. Characterization of persistent and sporadic *Listeria monocytogenes* strains by pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP). Syst. Appl. Microbiol. 26, 539–545.

Barancelli, G.V., Camargo, T.M., Reis, C.M.F., Porto, E., Hofer, E., Oliveira, C.A.F., 2011. Incidence of *Listeria monocytogenes* in cheese manufacturing plants from the northeast region of São Paulo, Brazil. J. Food Prot. 74, 816–819.

Barret, T.J., Gerner-Smidt, P., Swaminathan, B., 2006. Interpretation of pulsed-field gel electrophoresis patterns in foodborne disease investigations and surveillance. Foodborne Pathog. Dis. 3, 20–31.

Blodgget, R. Most Probable Number from Serial Dilutions. In: US Food and Drug Administration (FDA), Bacteriological Analytical Manual Online, 8th Edition, Modified from Revision A CD ROM version 1998 on 6/21/2000. Available in: <http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm109656.htm> Access in August 30, 2008.

Borges, M.F., 2006. Diagnóstico da contaminação por bactérias patogênicas em indústria de processamento de queijo de coalho e detecção de genes associados a fatores de virulência. Campinas, 2006. 199 f. Tese (Doutorado)—Faculdade de Engenharia de Alimentos. Universidade Estadual de Campinas, Campinas.

Brazil, 2002. Resolução RDC 275: Regulamento Técnico de Procedimentos Operacionais Padronizados aplicados aos Estabelecimentos Produtores/Industrializadores de Alimentos e a Lista de Verificação das Boas Práticas de Fabricação. Diário Oficial da União 206 (Seção I), 126–130.

Brito, J.R., Santos, E.M.P., Arcuri, E.F., Lange, C.C., Brito, M.A.V.P., Souza, G.N., Cerqueira, M.M.P.O., Soto Beltran, J.M., Call, J.E., Liu, Y., Porto-Fett, A.C.S., Luchansky, J.B., 2008. Retail survey of Brazilian milk and minas frescal cheese and a contaminated dairy plant to establish prevalence, relatedness, and sources of *Listeria monocytogenes* isolates. Appl. Environ. Microbiol. 74, 4954–4961.

Carvalho, J.D.G., Viotto, W.H., Kuaye, A.Y., 2007. The quality of Minas frescal cheese produced by different technological processes. Food Control 18, 262–267.

Casarotti, V.T., Gallo, C., Camargo, R., 1994. Ocorrência de *Listeria monocytogenes* em leite cru, leite pasteurizado tipo C e queijo minas frescal comercializados em Piracicaba-SP. Arch. Latinoam. Nutr. 44, 158–163.

Chambel, L., Sol, M., Fernandes, I., Barbosa, M., Zilhão, I., Barata, B., Jordan, S., Perni, S., Shama, G., Adrião, A., Faleiro, L., Requena, T., Peláez, C., Andrew, P.W., Tenreiro, R., 2007. Occurrence and persistence of *Listeria* spp. in the environment of ewe and cow's milk cheese dairies in Portugal unveiled by an integrated analysis of identification, typing and spatial–temporal mapping along production cycle. Int. J. Food Microbiol. 116, 52–63.

Chasseignaux, E., Toquin, M.T., Ragimbeau, C., Salvat, G., Colin, P., Ermel, G., 2001. Molecular epidemiology of *Listeria monocytogenes* isolates collected from the environment, raw meat and raw products in two poultry and pork-processing plants. J. Appl. Microbiol. 91, 888–899.

D'amico, D.J., Donnelly, C.W., 2009. Detection, isolation, and incidence of *Listeria* spp. in small-scale artisan cheese processing facilities: a methods comparison. J. Food Prot. 72, 2499–2507.

De Nes, F., Riboldi, G.P., Frazzon, A.P.G., d'Azevedo, P.A., Frazzon, J., 2010. Antimicrobial resistance and investigation of the molecular epidemiology of *Listeria monocytogenes* in dairy products. Rev. Soc. Bras. Med. Trop. 43, 382–385.

Destro, M.T., De Melo Serrano, A., Kabuki, D.Y., 1991. Isolation of *Listeria* species from some Brazilian meat and dairy products. Food Control 2, 110–112.

Evancho, G.M., Sveum, W.H., Moberg, L.J., Frank, J.F., 2002. Microbiological monitoring of the food processing environment, In: Downes, F.P., Ito, K. (Eds.), Compendium of Methods for the Microbiological Examination of Foods, forth ed. American Public Health Association, Washington, pp. 25–34.

Frye, C., Donnelly, C.W., 2005. Comprehensive survey of pasteurized fluid milk produced in the United States reveals a low prevalence of *Listeria monocytogenes*. J. Food Prot. 68, 973–979.

Fugett, E.B., Schoonmaker-Bopp, D., Dumas, N.B., Corby, J., Wiedmann, M., 2007. Pulsed-field gel electrophoresis (PFGE) analysis of temporally matched *Listeria monocytogenes* isolates from human clinical cases, foods, ruminant farms, and urban and natural environments revels source-associated as well as widely distributed PFGE types. J. Clin. Microbiol. 45, 865–873.

Graves, L.M., Hunter, S.B., Ong, A.R., Schoonmaker-Bopp, D., Hise, K., Kornstein, L., DeWitt, W.E., Hayes, P.S., Dunne, E., Mead, P., Swaminathan, B., 2005. Microbiological aspects of the investigation that traced the 1998 outbreak of listeriosis in the United States to contaminated hot dogs and establishment of molecular subtyping based surveillance for *Listeria monocytogenes* in the PulseNet network. J. Clin. Microbiol. 43, 2350–2355.

Graves, L.M., Swaminathan, B., Hunter, S.B., 2007. Subtyping *Listeria monocytogenes*, In: Ryser, E.T., Marth, E.H. (Eds.), *Listeria*, Listeriosis and Food Safety, third ed. CRC Press, Boca Raton, pp. 283–304.

Hamdi, T.M., Naim, M., Martin, P., Jacquet, C., 2007. Identification and molecular characterization of *Listeria monocytogenes* isolated in raw milk in the region of Algiers (Algeria). Int. J. Food Microbiol. 116, 190–193.

Hitchins, A.D., 2003. Detection and enumeration of *Listeria monocytogenes* in foods. Chapter 10, revised January US Food and Drug Administration (FDA), Bacteriological Analytical Manual Online (Available in: <http://www.cfsan.fda.gov/~ebam/bam-10.html>. Access in July 06, 2007).

Hofer, E., Reis, C.M.F., Hofer, C.B., 2006. Sorovares de *Listeria monocytogenes* e espécies relacionadas isoladas de material clínico humano. Rev. Soc. Bras. Med. Trop. 39, 32–37.

Kabuki, D.Y., Kuaye, A.Y., Wiedmann, M., Boor, K.J., 2004. Molecular subtyping and tracking of *Listeria monocytogenes* in latin-style fresh-cheese processing plants. J. Dairy Sci. 87, 2803–2812.

Kathariou, S., 2002. *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. J. Food Prot. 65, 1811–1829.

Kells, J., Gilmour, A., 2004. Incidence of *Listeria monocytogenes* in two milk processing environments, and assessment of *Listeria monocytogenes* blood agar for isolation. Int. J. Food Microbiol. 91, 167–174.

Larson, A.E., Johnson, E.A., Nelson, J.H., 1999. Survival of *Listeria monocytogenes* in commercial cheese brines. J. Dairy Sci. 82, 1860–1868.

Latorre, A.A., Van Kessel, J.A.S., Karms, J.S., Zurakowski, M.J., Pradhan, A.K., Zadoks, R.N., Boor, K.J., Schukken, Y.H., 2009. Molecular ecology of *Listeria monocytogenes*: evidence for a reservoir in milking equipment on a dairy farm. Appl. Environ. Microbiol. 75, 1315–1323.

Leite, P., Rodrigues, R., Ferreira, M., Ribeiro, G., Jacquet, C., Martin, P., Brito, L., 2006. Comparative characterization of *Listeria monocytogenes* isolated from Portuguese farmhouse ewe's cheese and from humans. Int. J. Food Microbiol. 106, 111–121.

Margolles, A., Mayo, B., de los Reyes-Gavilán, C.G., 1998. Polymorphism of *Listeria monocytogenes* and *Listeria innocua* strains isolated from short-ripened cheeses. J. Appl. Microbiol. 84, 255–262.

Martin, P., Jacquet, C., Goulet, V., Vaillant, V., De Valk, H., 2006. Pulsed-field gel electrophoresis of *Listeria monocytogenes* strains: the PulseNet Europe feasibility study. Foodborne Pathog. Dis. 3, 303–308.

Miettinen, M.K., Björkroth, K.J., Korkeala, H.J., 1999. Characterization of *L. monocytogenes* from an ice cream plant by serotyping and pulsed-field gel electrophoresis. Int. J. Food Microbiol. 46, 187–192.

Nero, L.A., de Mattos, M.R., Barros, M.A., Ortolani, M.B., Beloti, V., Franco, B.D., 2008. *Listeria monocytogenes* and *Salmonella* spp. in raw milk produced in Brazil: occurrence and interference of indigenous microbiota in their isolation and development. Zoonoses Public Health 55, 299–305.

Neves, E., Lourenço, A., Silva, A.C., Coutinho, R., Brito, L., 2008. Pulsed-field gel electrophoresis (PFGE) analysis of *Listeria monocytogenes* isolates from different sources and geographical origins and representative of the twelve serovars. Syst. Appl. Microbiol. 31, 387–392.

Pintado, C.M.B.S., Oliveira, A., Pampulha, M.E., Ferreira, M.A.S.S., 2005. Prevalence and characterization of *Listeria monocytogenes* isolated from soft cheese. Food Microbiol. 22, 79–85.

Pulsenet International, 2009. The international molecular subtyping network for foodborne disease surveillance. Standardized laboratory protocol for molecular subtyping of *Listeria monocytogenes* by Pulsed Field Gel Electrophoresis (PFGE). Available in http://www.pulsenetinternational.org/protocols/protocols.asp (Access in October 19, 2009).

Ragon, M., Wirth, T., Hollandt, F., Lavenir, R., Lecuit, M., Le Monnier, A., Brisse, S., 2008. A new perspective on *Listeria monocytogenes* evolution. PLoS Pathog. 4, 1–14.

Sauders, B.D., Sanchez, M.D., Rice, D.H., Corby, J., Stich, S., Fortes, E.D., Roof, S.E., Wiedmann, M., 2009. Prevalence and molecular diversity of *Listeria monocytogenes* in retail establishments. J. Food Prot. 72, 2337–2349.

Seeliger, H.P.R., Höhne, K., 1979. Serotyping of *Listeria monocytogenes* and related species. In: Bergan, T., Norris, J.R. (Eds.), Methods in microbiology, 13. Academic Press, London, pp. 31–49.

Silva, I.M.M., Almeida, R.C.C., Alves, M.A.O., Almeida, P.F., 2003. Ocurrence of *Listeria* spp. in critical control points and the environment of minas frescal cheese processing. Int. J. Food Microbiol. 81, 241–248.

Silva, N., Neto, R.C., Junqueira, V.C.A., Silveira, N.F.A., 2005. Manual de Métodos de Análise Microbiológica de Água. Varela, São Paulo.

Silva, N., Junqueira, V.C.A., Silveira, N.F.A., Taniwaki, M.H., Santos, R.F.S., Gomes, R.A.R., 2010. Manual de Métodos de Análise Microbiológica de Alimentos e Água, quarta ed. Varela, São Paulo.

Swaminathan, B., Gerner-Smidt, P., 2007. The epidemiology of human listeriosis. Microbes Infect. 9, 1236–1243.

Wagner, M., Eliskases-Lechner, F., Rieck, P., Hein, I., Allerberger, F., 2006. Characterization of *Listeria monocytogenes* isolates from 50 small-scale Austrian cheese factories. J. Food Prot. 9, 1297–1303.

Zaffari, C.B., Mello, J.F., Costa, M., 2007. Qualidade bacteriológica de queijos artesanais comercializados em estradas do litoral norte do Rio Grande do Sul, Brasil. Ciência Rural 37, 862–867.