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Selection of indigenous lactic acid bacteria presenting anti-listerial activity, and their role in reducing the maturation period and assuring the safety of traditional Brazilian cheeses

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ABSTRACT

Artisanal raw milk cheeses are highly appreciated dairy products in Brazil and ensuring their microbiological safety has been a great need. This study reports the isolation and characterization of lactic acid bacteria (LAB) strains with anti-listerial activity, and their effects on *Listeria monocytogenes* during refrigerated shelf-life of soft Minas cheese and ripening of semi-hard Minas cheese. LAB strains (n = 891) isolated from Minas artisanal cheeses (n = 244) were assessed for anti-listerial activity by deferred antagonism assay at 37 °C and 7 °C. The treatments comprised the production of soft or semi-hard Minas cheeses using raw or pasteurized milk, and including the addition of selected LAB only [*Lactobacillus brevis* 2-392, *Lactobacillus plantarum* 1-399 and 4 *Enterococcus faecalis* (1-37, 2-49, 2-388 and 1-400)], *L. monocytogenes* only, selected LAB co-inoculated with *L. monocytogenes*, or without any added cultures. At 37 °C, 48.1% of LAB isolates showed anti-listerial capacity and 77.5% maintained activity at 7 °C. Selected LAB strains presented a bacteriostatic effect on *L. monocytogenes* in soft cheese. *L. monocytogenes* was inactivated during the ripening of semi-hard cheeses by the mix of LAB added. Times to attain a 4 log-reduction of *L. monocytogenes* were 15 and 21 days for semi-hard cheeses produced with raw and pasteurized milk, respectively. LAB with anti-listerial activity isolated from artisanal Minas cheeses can comprise an additional barrier to L. monocytogenes growth during the refrigerated storage of soft cheese and help shorten the ripening period of semi-hard cheeses aged at ambient temperature.

1. Introduction

Modern consumers seek out traditional foods, and there is widespread awareness that such foods can be an instrument of economic recovery, providing local resources and access to employment, which contributes to enhancement of local economic growth, entrepreneurship and innovation (Borelli et al., 2011). Minas cheeses are among the most popular artisanal cheeses consumed in Brazil. These cheeses are produced in the state of Minas Gerais, and include soft (i.e., Frescal), semi-hard and hard types (Padrão, Meia-cura, Serro, Canastra, etc.). Artisanal Minas cheeses have been manufactured by small farmers in a traditional, empirical manner using raw milk and indigenous lactic acid bacteria (LAB) for over 200 years (Borelli et al., 2006). Estimates indicate that 220,000 tons/year of artisanal cheese are produced in 7 regions of Minas Gerais state: Araxá, Canastra, Campo das Vertentes, Cerrado, Serro, Serra do Salitre and Triângulo Mineiro (Milkpoint, 2017).

While artisanal raw milk cheeses possess highly desirable organoleptic properties, it is also well documented that some may be produced without the benefit of sanitary inspections, using inadequate manufacturing practices and sold without appropriate

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packaging via an inefficient cold chain (Cavalcante et al., 2013; Raimundo, 2013; Vasconcelos and Marin, 2008). For instance, Perin et al. (2017) observed high numbers of coagulase positive cocci and coliforms in artisanal cheese samples produced in different regions of Minas Gerais state, Brazil. Brazilian legislation stipulates that raw milk cheeses can be sold only after ripening for 60 days at or above 5 °C (MAPA, 2000). However, it is also well known that artisanal Minas cheeses are typically ripened for only 17–22 days (Milkpoint, 2017) in order to retain their typical sensory characteristics, which may lead to producers operating outside the law and endangering consumer health (Dores and Ferreira, 2012). A new (2011) Brazilian legislation sought to fix this issue by allowing the sale of cheeses with a reduced (<60 days) ripening time (MAPA, 2011), as long as the ripening time was determined by specific research studies and approved by the Ministry of Agriculture. The research presented here is designed to investigate suitable minimum ripening period for Brazilian artisanal cheeses and study effects of aging on pathogenic microbiota.

Listeria monocytogenes is a bacterial pathogen commonly linked with ready-to-eat products, including cheeses. *L. monocytogenes* is the causative agent of listeriosis, which can lead to gastrointestinal diseases or septicemia, abortions, and often leads to death of immunocompromised patients and susceptible individuals such as, pregnant women, newborns and elderly (Sip et al., 2012). Different studies in Brazil have shown that indigenous LAB isolated from cheeses showed ability to inhibit *L. monocytogenes* (Alexandre et al., 2002; Cavicchioli et al., 2017; Guedes Neto et al., 2005; Ortolani et al., 2010; Tulini et al., 2013). The mechanisms of this antagonism are not completely elucidated, as LAB can produce several antimicrobial compounds, including lactic acid, hydrogen peroxide, diacetyl, reuterin and bacteriocins (Guillier et al., 2008). Artisanal Minas cheeses are often produced with the addition of “pingo”, a fermented whey collected from the previous cheese production containing the indigenous LAB needed for the development of the cheeses organoleptic properties. LAB from “pingo” may also be responsible for inhibition or inactivation of *L. monocytogenes* and other foodborne pathogens, resulting in the reduction of ripening time and comprising an appropriate foodborne pathogen risk management measure.

Refrigerated soft Minas cheeses showed the highest incidence of *L. monocytogenes* (3–45% in Brito et al., 2008; Carvalho, 2003; Silva et al., 1998), likely due to its relatively high moisture (55–58%) and pH (5.0–6.3) and relatively low salt content (1.4–1.6%) (Malheiros et al., 2012a). *L. monocytogenes* has been less frequently isolated from semi-hard Minas cheeses (1.4–6% in Raimundo, 2013; Silva et al., 1998; Souza, 2006), likely because of interaction with the natural microbiota, lower pH and water activity, and higher salt content, all of which continue to change during ripening. There are few papers regarding the application of LAB strains as antagonistic agents against *L. monocytogenes* in fresh or soft cheeses produced in Brazil (Jesus et al., 2016; Nascimento et al., 2008; Pingitore et al., 2012), and none of them considered cheeses produced with raw milk or Brazilian artisanal cheeses, including the semi-hard type typically produced in Minas Gerais. The objective of this study was to isolate and characterize LAB strains with anti-listerial activity and further model their effects on *L. monocytogenes* during chilled shelf-life of soft Minas cheese and ripening of semi-hard Minas cheese manufactured with raw and pasteurized milk.

2. Material and methods

2.1. LAB recovery from artisanal Minas cheeses

Samples of artisanal Minas cheeses (n = 244) were collected in the state of Minas Gerais, Brazil, between July/2014 and February/2015, from five cheese producing regions: Araxá, Campo das Vertentes, Canastra, Cerrado and Serro (n = 55, 51, 46, 43 and 49, respectively). LAB strains were isolated from cheese samples using the methodology of Njongmeta et al. (2015). Briefly, after dilution, aliquots were pour plated in MRS agar (de Man, Rogosa and Sharpe, EMD Millipore Corporation, Billerica/MA), a selective medium for enumeration and isolation of lactobacilli, and M17 agar (HiMedia Laboratories, Mumbai/India), a non-selective medium for enumeration and isolation of lactococci, overlayed with 1.2% bacteriological agar (InLab – Alamar Tecno-Científica Ltda., São Paulo/SP/Brazil), following incubation at 30 °C for 48 h. Five typical colonies on MRS and M17 agar were selected for purification, following additional incubation at 30 °C for 48 h in the respective media. Gram (Gregersen, 1978) and catalase (3% hydrogen peroxyde) tests as well as morphologic observation, were conducted in order to eliminate non-LAB isolates (e.g. Gram-positive, catalase-negative and cocci or rod morphology). Cultures presumptively identified as LAB were maintained in MRS broth (Acumedia, Neogen Corporation, Lansing/MI) with 30% glycerol at −80 °C.

2.2. L. monocytogenes strains and preparation of cell suspensions

L. monocytogenes strain 3968 - serotype 1/2b isolated from cheese (LM 3968) and *L. monocytogenes* strain 3973 - serotype 4b isolated from raw milk (LM 3973), both kindly donated by Oswaldo Cruz Foundation (Rio de Janeiro/RJ/Brazil) were used for all experiments. Each L. *monocytogenes* strain was cultured and cell suspensions (10^8 CFU/mL) were prepared according to Sant'Ana et al. (2012a).

2.3. Screening of LAB for anti-listerial capacity

Anti-listerial capacity of selected LAB strains was determined by the deferred antagonism assay of Harris et al. (1989) with some modifications. Each LAB strain was cultured separately overnight (30 °C, 18–24 h), spotted (1–3 μL) onto the surface of MRS or M17 agar plates (for LAB strains isolated in MRS and M17 agar, respectively), following incubation at 30 °C until evident growth (18–24 h). Inoculated plates were covered with 10 mL of BHI soft agar (Brain Heart Infusion - BHI broth, EMD Millipore Corporation, Billerica/MA, added of 0.75% bacteriological agar) seeded with *L. monocytogenes* culture (10^6–10^7 CFU/mL). Strains LM 3968 or LM 3973 were tested separately. After inoculating plates at 37 °C overnight, zones of inhibition of *L. monocytogenes* were inspected qualitatively by absence or presence. LAB strains that presented anti-listerial capacity at 37 °C were also tested at refrigeration temperature (7 °C, 10 days).

2.4. LAB proteolytic and acidifying capacity

Proteolytic activity and acidifying capacity were assayed according to the methodologies of Franciosi et al. (2009) and Durlu-Ozkaya et al. (2001) respectively, for any LAB strains presenting anti-listerial activity at 7 °C and 37 °C. The LAB strains were expected to decrease the pH to 5.3 after 6 h at 30 °C due to acid production (Beresford et al., 2001) to be considered suitable for use as a starter culture. On the other hand, proteolytic activity was confirmed qualitatively by clear zones around the LAB colonies onto the surface of milk agar.

2.5. Identification of selected LAB strains

Six LAB strains (3 isolated from MRS agar and 3 from M17 agar) with anti-listerial capacity at 7 °C and 37 °C, acidifying and proteolytic activities, and belonging to the same Minas Gerais state

region were selected for further cheese production and identified by PCR. Sample preparation, sequencing and bioinformatics analysis were performed by Neoprospecta Microbiome Technologies (Florianópolis/SC/Brazil). The rRNA 16S V3/V4 region was amplified using the 341F (CCTACGGGRSGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) primers (Wang and Quian, 2009; Caporaso et al., 2011). OTU Picking was performed using Blastn 2.2.28 (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Only sequences with hits of 99% of identity in an alignment covered over 99% were considered to determine taxonomy (Christoff et al., 2017).

2.6. Soft and semi-hard Minas cheese production

Before each cheese production, preparation of six identified LAB strains with known anti-listerial activity included their separate cultivation in MRS broth at 30 °C for 24 h, followed by two consecutive inoculations. The third inoculation was carried out in 90 mL of MRS broth. LAB concentration was determined using a McFarland scale (McFarland turbidimeter, MS Tecnopon, Piracicaba/SP/Brazil), where a 1.00 reading corresponded to 3×10^8 CFU/mL. Preparation of L. *monocytogenes* strains (strains LM 3968 and LM 3973) was the same as described above.

Sixteen different treatments were performed in duplicate, conducted in different days, and consisted of production of soft or semi-hard Minas cheeses using raw or pasteurized milk, and including the addition of selected LAB alone, *L. monocytogenes* alone, selected LAB co-inoculated with *L. monocytogenes*, or without any added cultures. All treatments were assumed to contain naturally occurring microbiota (e.g. indigenous LAB). Additional LAB (as a pool) were added in a rate of 10^6–10^7 CFU/mL of milk, while the two *L. monocytogenes* strains (also as a pool) were added at 10^5–10^6 CFU/mL of milk to simulate massive contamination.

The laboratory-based production of Soft Minas cheese was adapted from Malheiros et al. (2012b). Ten liters of raw or pasteurized milk, purchased at a dairy farm (Araras/SP/Brazil), were heated to 34 ± 1 °C and added with 5 mL of $CaCl_2$ (saturated solution), 9 mL of commercial rennet Estrella (85% bovine pepsin +15% bovine chymosin, Chr. Hansen, Valinhos/SP/Brazil) and selected LAB and/or *L. monocytogenes* strains, depending upon the treatment. After 40 min coagulation, curd cutting, slight agitation and resting for 30 min, sodium chloride (2 g/L) was added and curd was allowed to rest for another 30 min. The whey was drained and the curd was placed into perforated sterile cylindrical shapes. Cheeses were kept at room temperature for 1 h for dripping, turned and left for an additional 1 h for final dripping. Unmolded cheeses were packed in plastic bags, following storage at 7 ± 1 °C for 15 days.

Semi-hard Minas cheese production was based on Rodríguez et al. (2005) with some modifications. Initial steps of production were the same as for soft cheese until the first 30 min resting. Whey was then drained off and the curd was distributed into perforated sterile cylindrical molds. Cheeses were maintained at room temperature for 1 h for dripping, turned and pressed for 1 h. Cheese surfaces were dry salted, and the cheeses were placed on wooden shelves and ripened at 22 ± 1 °C for 22 days. Cheeses were turned daily during ripening.

2.7. Microbiological analysis of soft and semi-hard cheeses

Microbiological analysis of soft and semi-hard cheeses during refrigerated shelf-life and ripening, respectively, included LAB and *L. monocytogenes* counting, and was carried out according to Njongmeta et al. (2015) and Ryser and Donnelly, 2015 respectively, at appropriate intervals. For soft cheese, analysis were performed on day 0 (immediately after production), days 1, 2, 3 and 4 (twice a day, early morning and late afternoon) and days 5, 7, 9, 12 and 15 (once a day); while for semi-hard cheese, analysis occurred on day 0 (immediately after production and after 4, 8, 12, 16 and 20 h) and on days 2, 3, 4, 8, 12, 16, 19 and 22 (once a day). Also, cheese pH and temperature were measured in non-*L. monocytogenes* inoculated cheeses using a portable pH meter coupled with knife electrode and temperature sensor (AK103 pHmeter, SC18 electrode, Akso Eletronic Products Ltda., São Leopoldo/RS/Brazil).

2.8. Growth potential (δ) of LAB and L. monocytogenes

Growth potential (δ) of LAB and *L. monocytogenes* in each treatment of soft and semi-hard Minas cheeses was determined by calculating the difference between microbial counts (in log CFU/g) at the end of refrigerated shelf-life (15 days) for soft cheese or ripening period for semi-hard cheese (22 days) and the beginning (day 0). Cheeses were considered to support *L. monocytogenes* growth when δ was higher than 0.5 log CFU/g (Jesus et al., 2016; Sant'Ana et al., 2012b, 2013). When δ values were negative or lower than 0.5 log CFU/g, cheeses were considered not to support *L. monocytogenes* growth.

2.9. Statistical analysis

2.9.1. Modeling the growth/inactivation data

Primary modeling of growth/inactivation log-transformed data for LAB and *L. monocytogenes* was conducted. The DMFit add-in of Excel® 2010 (Microsoft, Redmond/WA) was used to fit the Baranyi and Roberts (1994) model to the counts of LAB and *L. monocytogenes* aiming to estimate their maximum growth rate (μ_{max}, expressed as 1/h). Inactivation rate (k_{max}, expressed as 1/h) was calculated using the Bigelow and Esty (1920) log-linear model through the GInaFiT Excel add-in software (Geeraerd and Van Impe Inactivation Model Fitting Tool, Geeraerd et al., 2005).

2.9.2. Correspondence analysis for LAB

Correspondence analysis was applied for the selection of LAB strains with anti-listerial capacity and technological properties to be used in cheese production ($P \leq 0.05$). This analysis was conducted using XLSTAT® version 2017.02.43733 software (Addinsoft, Paris/France) as an Excel add-in. Significant statistical differences ($P \leq 0.05$) were checked for initial and final bacterial counts, growth potential, maximum growth/inactivation rates and pH employing one-factor analysis of variance (ANOVA) followed by Duncan's test conducted in Assistat version 7.7 software (Campina Grande/PB/Brazil) (Silva and Azevedo, 2002) for each cheese type. Model fit was also assessed by coefficient of determination (R^2).

3. Results

3.1. Overall results for anti-listerial capacity and technological properties

Anti-listerial capacity and technological properties of LAB strains according to isolation medium and Minas Gerais cheese production regions are detailed in Table 1. A total of 891 LAB strains (466 from MRS agar and 425 from M17 agar) were isolated from Minas artisanal cheeses samples. Anti-listerial tests at 37 °C showed that 73.0% and 70.8% of LAB isolated in MRS agar presented antagonism against LM 3968 and LM 3973, respectively. On the other hand, the percentage of LAB isolated in M17 agar that antagonism against LM 3968 and LM 3973 were 21.2% and 23.1%, respectively. When LAB strains with anti-listerial activity at 37 °C against both *L. monocytogenes* strains were tested at 7 °C, 91.5% and 63.4% of LAB isolated in MRS and M17 agar were able to keep their

Table 1
Number of lactic acid bacteria (LAB) isolates per cheese producing region and percentage presenting anti-listerial capacity at 37 °C and 7 °C, and proteolytic and acidifying activities.

Isolation medium	Cheese producing region	Number of LAB isolates initially	Anti-listerial capacity at 37 °C (%)[a]		Anti-listerial capacity at 7 °C (%)[a,b]		Number of LAB isolates after anti-listerial tests	Proteolytic activity (%)	Acidifying activity (%)
			LM 3968	LM 3973	LM 3968	LM 3973			
MRS agar	Araxá	113	73.5	77.0	88.0	85.1	73	24.7	2.7
	Campo das Vertentes	82	64.6	65.9	88.7	87.0	47	42.6	6.4
	Canastra	107	71.0	60.7	84.2	98.5	64	48.4	43.8
	Cerrado	99	76.8	74.7	97.4	100	74	14.9	13.5
	Serro	65	80.0	76.9	92.3	96.0	48	29.2	6.3
M17 agar	Araxá	98	13.3	16.3	84.6	68.8	11	45.5	36.4
	Campo das Vertentes	101	29.7	28.7	66.7	69.0	20	60.0	65.0
	Canastra	99	23.2	27.3	73.9	63.0	17	35.3	23.5
	Cerrado	22	36.4	18.2	50.0	100	4	25.0	50.0
	Serro	105	15.2	21.0	43.8	36.4	7	0	0

[a] Percentage of LAB strains that caused inhibition of Listeria monocytogenes 3968 serotype 1/2b (LM 3968) and L. monocytogenes 3973 serotype 4b (LM 3973) (assessed by the presence of inhibition zones).
[b] Percentage of LAB isolates with anti-listerial capacity at 37 °C and able to maintain the anti-listerial activity at 7 °C.

anti-listerial activity, respectively.

It is notable that LAB isolated in MRS agar incubated at 37 °C presented higher anti-listerial capacity (vs. M17 agar isolates) no matter the cheese production region. Additionally, 91.1% of LAB isolated in MRS agar presented an antagonistic activity against both *L. monocytogenes* strains, while 49.6% of LAB strains isolated in M17 agar presented such activity. When proteolytic capacity was assayed 30.7% and 40.7% of LAB isolated in MRS and M17 agar were able to degrade casein in milk agar, respectively. When acidifying activity was measured, 15.0% and 39.0% of LAB from MRS and M17 could reduce the pH below 5.3 and were considered good acidifiers, respectively. The proteolytic capacity and acidifying activity of LAB strains isolated were quite variable among regions. Despite this, a highest percentage of LAB isolated in MRS from cheese produced in the Canastra region showed the proteolytic capacity and acidifying activity. On the other hand, when isolation was done on M17 agar, the highest percentage of LAB presenting proteolytic and acidifying activities originated from cheeses produced in the Campo das Vertentes region.

3.2. Correspondence analysis and identification of LAB strains

Correspondence analysis elucidated the relationship between LAB isolated from different cheese producing regions, anti-listerial capacity (two temperatures × two *L. monocytogenes* strains) and acidifying and proteolytic abilities (Fig. 1A). Correspondence analysis outputs showed for LAB isolated on MRS that first and second dimensions successfully encompassed 86.5% and 12.5%, respectively, explaining 99.0% of the total variance in the data set, with a significant difference between variables analyzed ($P < 0.0001$). A large cluster encompassing the regions of Araxá and Serro and anti-listerial capacity against both *L. monocytogenes* strains at both temperatures is evident in Fig. 1A. LAB strains from the regions of Campo das Vertentes, Canastra and Cerrado associate around the Araxá and Serro cluster. LAB strains from the Canastra region associate near acidification ability, while strains from the Campo das Vertentes region associate near proteolytic ability.

Correspondence analysis for LAB strains isolated on M17 agar is shown in Fig. 1B. The first two (of four) dimensions comprised 94.3% of the total inertia observed. The first and second dimensions comprise 79.8% and 14.5% respectively of the total inertia observed, with significant differences between variables investigated ($P < 0.0001$). There was an interesting subset of categories, highlighting a specific association between Canastra region and anti-listerial activity for LM 3968 at 7 °C and for LM 3973 at 7 °C and 37 °C. There was also a subset of categories including Cerrado region and anti-listerial activity for LM 3968 at 37 °C. Campo das Vertentes region and proteolysis capacity, as well as Araxá region and acidification capacity, could also be considered subsets, although the distance between points was larger.

Six LAB strains belonging to Canastra region were chosen for PCR identification, based on the correspondence analysis. The selected LAB strains were identified as *Lactobacillus brevis*2-392, *Lactobacillus plantarum* 1-399 (both isolated on MRS) and 4 strains of *Enterococcus faecalis* (one isolated from MRS and 3 from M17) (1-37, 2-49, 2-388 and 1-400). The isolation of *Enterococcus* strains is not completely unexpected because although M17 (and to a lesser extend MRS) agars are generally used to select *Lactobacillus* and *Lactococcus* spp., they are non-selective so a variety of other LAB genera (including *Enterococcus* spp.) may occasionally be isolated.

3.3. Modeling the growth/inactivation data

The primary growth or inactivation models were fitted to LAB and *L. monocytogenes* counts to describe their behavior during refrigerated shelf-life of soft cheese and ripening period of semi-hard cheese (Fig. 2). LAB presented a growth pattern in all treatments studied. Initial concentrations of LAB in soft and semi-hard cheeses with only the indigenous microbiota ranged from 2.47 to 4.39 log CFU/g and from 3.62 to 5.16 log CFU/g, respectively; and from 7.27 to 8.08 log CFU/g and from 7.40 to 7.59 log CFU/g for cheeses prepared with selected LAB, respectively ($P < 0.0001$). LAB did not show evidence of a lag phase in any treatment. Final LAB concentrations varied from 6.62 to 10.71 log CFU/g, and treatments with addition of selected LAB differed significantly from those with no LAB addition ($P < 0.0001$). Average pseudo-R^2 values for primary modeling of LAB growth curves were 0.89 and 0.92 for soft and semi-hard cheeses, respectively (data not shown).

Growth potential (δ) and maximum growth (μ_{max}) or inactivation (k_{max}) rates are shown in Table 2. All LAB presented δ greater than 0.5 log UFC/g, and values of treatments containing only the natural microbiota with/without *L. monocytogenes* addition were generally significantly higher than those with deliberate addition of LAB ($P < 0.05$). The cheeses with added LAB showed higher and faster decreasing in pH (Fig. 3), although final values were not significantly different for semi-hard cheeses ($P > 0.05$). LAB presented higher μ_{max} in soft and semi-hard cheeses containing natural microbiota and *L. monocytogenes*, although the difference for semi-hard cheeses was not statistically significant ($P > 0.05$).

L. monocytogenes was able to grow in soft cheeses even with the

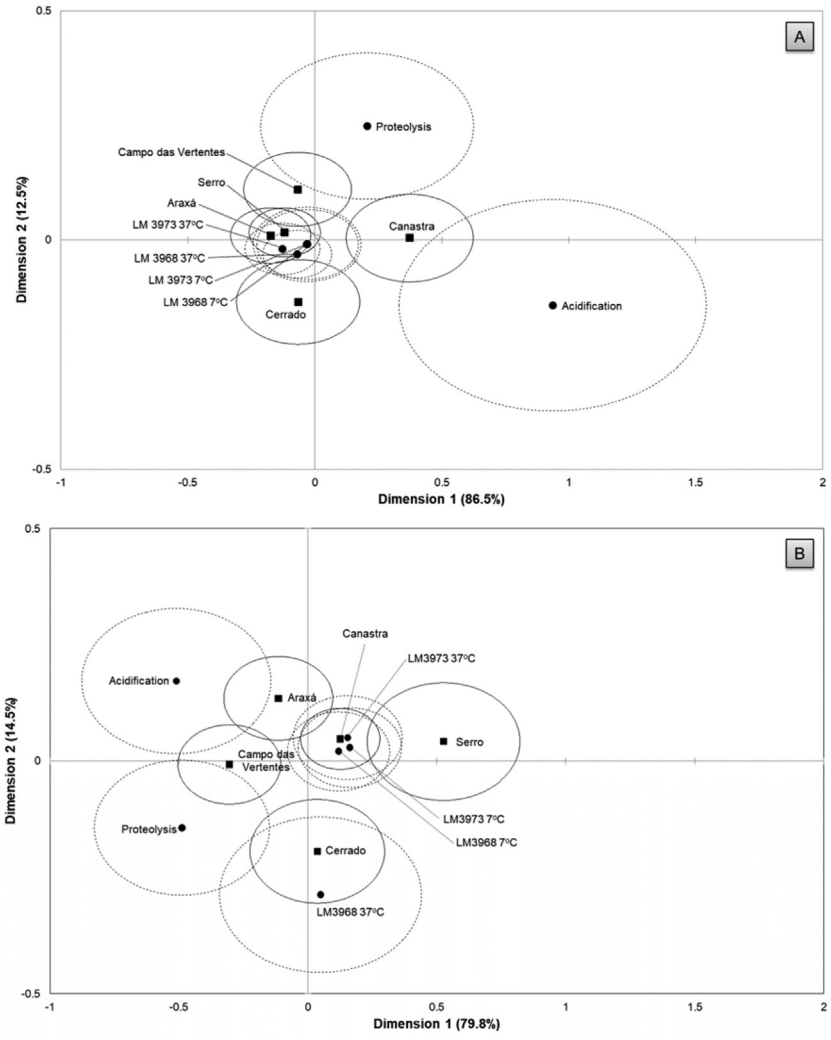

Fig. 1. Map of correspondence analysis of lactic acid bacteria (LAB) isolated in MRS agar (A) and M17 agar (B). LAB strains were originated from five cheese producing regions of Minas Gerais state (Araxá, Campo das Vertentes, Canastra, Cerrado and Serro) – represented by squares and solid lines. LAB anti-listerial capacity at 37 °C and 7 °C against two *Listeria monocytogenes* strains (LM 3968 – serotype 1/2b and LM 3973 – serotype 4b) and proteolytic and acidifying ability – represented by circles and dashed lines.

presence of deliberately added LAB strains. However, the *L. monocytogenes* δ in these treatments was significantly smaller compared to those containing only the natural microbiota (Table 2) ($P < 0.05$), suggesting a bacteriostatic effect of added LAB. *L. monocytogenes* could grow in semi-hard cheeses for treatments with natural microbiota, and growth was significantly higher in cheese produced with pasteurized milk ($P > 0.05$). When selected LAB were deliberately added, *L. monocytogenes* concentration was reduced by about 4 log CFU/g in pasteurized milk cheese, and were inactivated below the limit of detection of 100 CFU/g in raw milk cheese (reduction of at least 5.81 log CFU/g). Times for reducing 4 log cycles of *L. monocytogenes* in semi-hard cheeses produced with raw and pasteurized milk were 356 h (almost 15 days) and 507 h (around 21 days), respectively. *L. monocytogenes* growth curves in soft and semi-hard cheeses showed an average pseudo-R^2 of 0.81 and 0.85, respectively; while inactivation curves in semi-hard cheeses had average pseudo-R^2 of 0.94 (data not shown). There were no significant differences regarding *L. monocytogenes* μ_{max} in soft cheese ($P > 0.05$), while the values of k_{max} for this pathogen differed significantly in semi-hard cheese (Table 2).

4. Discussion

Isolation of LAB strains with anti-listerial activity from cheese samples is quite common around the world. Our results showed that 48.1% of 891 LAB isolated from artisanal Minas cheeses presented anti-listerial capacity, which is higher than both the results of Alexandre et al. (2002), who observed that 15.1% of 192 LAB isolates from cheeses of Serro region were antagonistic against *L. monocytogenes* Scott A; and those of Ortolani et al. (2010), who found that 14.9% of 389 LAB strains presented anti-listerial activity in samples from raw milk and soft cheese in Minas Gerais state. Distinct percentages of LAB with *L. monocytogenes* antagonistic properties may be a result of different sampling methodologies, antagonism targeted (i.e., general or bacteriocin-based, for instance), composition of culture media, culturing methods and strategy for antagonism assessment (i.e., qualitative × quantitative). The mechanisms of *L. monocytogenes* inhibition may also vary among each LAB strain and occur to different extent, as affected by decreased pH levels, competition for nutrients and different antimicrobial compounds produced, including bacteriocins (Parente and Ricciardi, 1999).

Our results also demonstrated that a great number of LAB

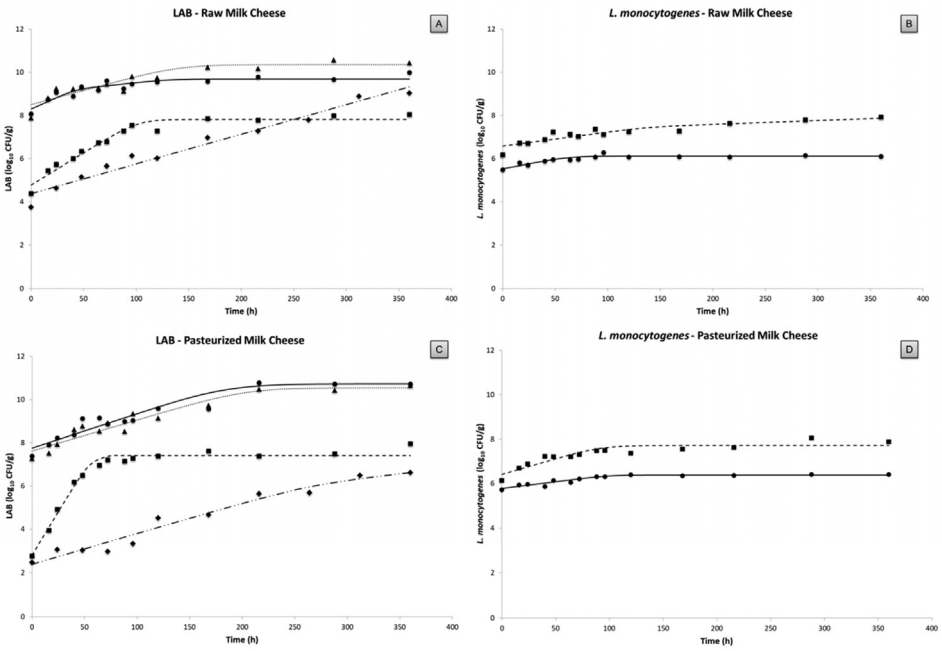

Fig. 2. Evolution of lactic acid bacteria (LAB) and *L. monocytogenes* (LM) in soft (A–D) and semi-hard (E–H) Minas cheeses produced with raw or pasteurized milk (A–H). Presence of naturally occurring microbiota, e.g. indigenous LAB (NM), was considered in all treatments. Points represent the experimental data whereas lines represent the outputs of Baranyi and Roberts (1994) model fitting, when growth was observed, and Bigelow and Esty (1920) Log-linear Regression model fitting, when inactivation was observed. Treatments: ◆ — · · NM; ▲ ····· NM + LAB; ■ - - - NM + LM; and ● —— NM + LAB + LM.

Table 2
Growth potential (δ) and predicted maximum growth (μ_{max})/inactivation (k_{max}) rates of lactic acid bacteria (LAB) and L. monocytogenes (LM) in soft and semi-hard Minas cheeses.

Type of Milk	Treatments[a,b]	LAB δ (log CFU/g)	LAB μ_{max} (1/h)	LM δ[c] (log CFU/g)	LM μ_{max} or LM k_{max} (1/h)	Initial pH (day 0)	Ending pH (day 15 or 22)	4 log CFU/g reduction time (days)
Soft Minas Cheese								
Raw	NM	5.28 ± 0.16^{a}	0.0137 ± 0.0011^{c}	–	–	6.69 ± 0.25^{a}	5.80 ± 0.30^{b}	–
	NM + LAB	2.55 ± 0.83^{cd}	0.0131 ± 0.0015^{c}	–	–	6.50 ± 0.03^{a}	4.87 ± 0.25^{c}	–
	NM + LM	3.65 ± 0.36^{b}	0.0297 ± 0.0012^{b}	1.75 ± 0.09^{a}	0.0065 ± 0.0042^{a}	–	–	–
	NM + LAB + LM	1.91 ± 0.05^{d}	0.0215 ± 0.0150^{bc}	0.61 ± 0.02^{b}	0.0087 ± 0.0008^{a}	–	–	–
Pasteurized	NM	4.15 ± 0.10^{b}	0.0141 ± 0.0043^{c}	–	–	6.75 ± 0.03^{a}	6.64 ± 0.09^{a}	–
	NM + LAB	3.39 ± 0.02^{bc}	0.0144 ± 0.0003^{c}	–	–	6.36 ± 0.21^{a}	5.19 ± 0.05^{c}	–
	NM + LM	5.19 ± 0.37^{a}	0.0801 ± 0.0021^{a}	1.75 ± 0.13^{a}	0.0141 ± 0.0049^{a}	–	–	–
	NM + LAB + LM	3.33 ± 0.30^{bc}	0.0160 ± 0.0014^{c}	0.68 ± 0.15^{b}	0.0061 ± 0.0002^{a}	–	–	–
P-value		0.0002	<.0001	0.0007	0.1957	0.2078	0.0035	–
Semi-hard Minas Cheese								
Raw	NM	3.69 ± 0.48^{c}	0.0158 ± 0.0004^{a}	–	–	6.47 ± 0.08^{a}	5.53 ± 0.60^{a}	–
	NM + LAB	2.93 ± 0.33^{d}	0.0157 ± 0.0099^{a}	–	–	6.37 ± 0.31^{a}	5.10 ± 0.39^{a}	–
	NM + LM	3.19 ± 0.40^{cd}	0.1348 ± 0.0103^{a}	0.85 ± 0.41^{b}	0.0534 ± 0.0243^{a}	–	–	–
	NM + LAB + LM	3.18 ± 0.19^{cd}	0.0263 ± 0.0068^{a}	-5.81 ± 0.05^{d}	-0.0260 ± 0.0008^{b}	–	–	14.9
Pasteurized	NM	6.11 ± 0.04^{a}	0.0161 ± 0.0035^{a}	–	–	6.76 ± 0.04^{a}	5.47 ± 0.06^{a}	–
	NM + LAB	2.94 ± 0.08^{d}	0.0249 ± 0.0037^{a}	–	–	6.51 ± 0.04^{a}	4.86 ± 0.07^{a}	–
	NM + LM	4.83 ± 0.24^{b}	0.0990 ± 0.0950^{a}	1.93 ± 0.56^{a}	0.0637 ± 0.0041^{a}	–	–	–
	NM + LAB + LM	3.20 ± 0.13^{cd}	0.0857 ± 0.0418^{a}	-4.05 ± 0.29^{c}	-0.0182 ± 0.0013^{b}	–	–	21.1
P-value		<.0001	0.0622	<.0001	0.0036	0.2518	0.3387	–

[a-d] Within a column, means without a common superscript differ significantly accordingly Duncan's test ($P \leq 0.05$).
[a] Treatments included cheese production with raw or pasteurized milk and inoculation with LAB with anti-listerial activity (LAB) alone, LM alone (LM) and LAB co-inoculated with LM (LAB + LM). For all treatments, presence of natural microbiota (NM) was always considered.
[b] Values expressed as mean ± standard deviation of duplicates.
[c] Negative δ values indicate that LM was inactivated.

strains with antagonistic capacity against *L. monocytogenes* at 37 °C were able to inhibit this pathogen also at 7 °C, although it was quite variable for M17 isolates from all regions. The ability to maintain anti-listerial activity at different temperatures, especially at refrigeration temperatures, is very important since *L. monocytogenes* can grow or survive in a wide temperature range (from 1 to 45 °C, Melo et al., 2015). Soft cheeses are generally stored at refrigeration temperatures and the psychrotrophic nature of *L. monocytogenes*, combined with other factors (high water activity of these cheeses, etc.) enhances its growth and/or survival in this type of product. Numerous outbreaks involving *L. monocytogenes* in soft cheeses have been reported (Bille et al., 2006; Jackson et al., 2011; Koch et al., 2010; MacDonald et al., 2005; Makino et al., 2005); so, the search for anti-listerial LAB effective in soft cheeses

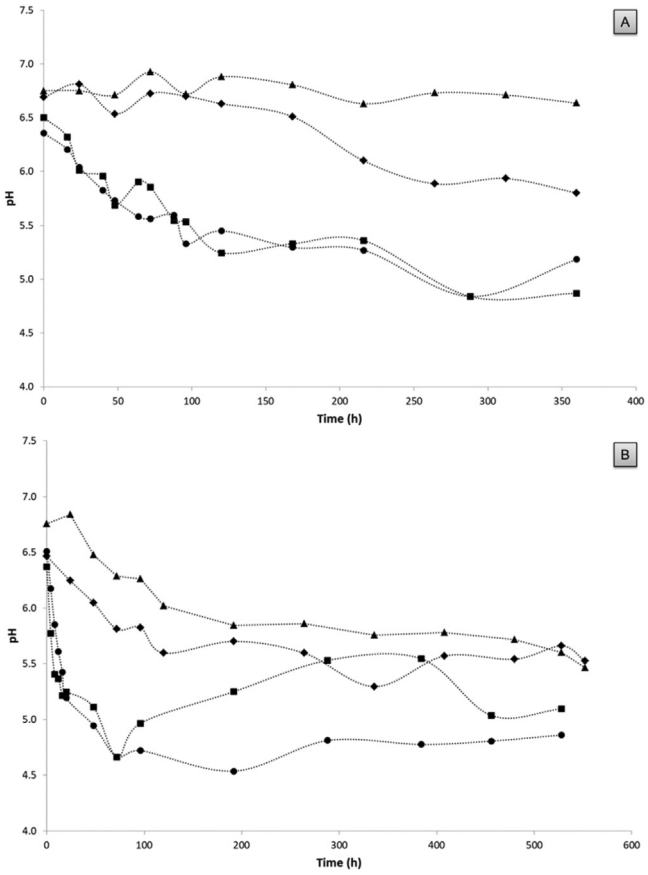

Fig. 3. pH profile during refrigerated shelf-life of soft cheese (A) or ripening of semi-hard cheese. Treatments included the production of cheese with raw or pasteurized (Past) milk with addition or not of selected lactic acid bacteria (LAB) strains with anti-listerial activity. Presence of naturally occurring microbiota, e.g. indigenous LAB (NM), was considered in all treatments. Treatments: ◆ Raw milk + NM; ■ Raw milk + NM + LAB; ▲ Pasteurized milk + NM; and ● Pasteurized milk + NM + LAB.

at multiple temperatures is crucial.

Technological properties, like proteolytic and acidifying capacities, can support the selection of LAB for application as starter or adjunct cultures in cheese production. LAB that were able to reduce the pH below 5.3 and presented proteolytic activity were selected in this study. The ability of LAB to rapidly produce acid is a very important property in fermented products, including cheeses, since a rapid pH drop is essential for coagulation, curd firmness and control of undesirable contaminants (Ribeiro et al., 2014). A pH decrease as a result of lactic acid production may be enough to inhibit some bacterial pathogens. Thus, controlling acidification in the initial steps of cheese-making is also of chief importance to successfully limiting *L. monocytogenes* growth (Millet et al., 2006). Casein degradation play a vital role in texture development in cheese and, some peptides can contribute to aroma development. Intracellular peptidases and proteinases released from bacterial cell walls after cell lysis also help in casein hydrolysis (Sarantinopoulos et al., 2001).

The selection of LAB strains with anti-listerial and technological properties was based on a correspondence analysis carried out. According to Greenacre (2007), correspondence analysis can help to understand complex patterns in the data and to generate hypotheses that can be tested in subsequent stage of research. The six LAB strains isolated from Minas cheeses produced in the Canastra region, identified as *Lb. Brevis* 2-392, *Lc. plantarum* 1-399 and *E. faecalis* (1-37, 2-49, 2-388 and 1-400) were chosen for the further step of this study. The selection of these species for addition in the cheeses is consistent with the findings of Nóbrega (2007) that reported that *Lactococcus*, *Streptococcus, Enterococcus, Lactobacillus* and *Leuconostoc* are the main LAB genera found in artisanal Minas cheeses produced in the Canastra region. Resende et al. (2011) found *Lactobacillus* as the most frequent LAB genus in Canastra cheeses, being *Lb. rhamnosus* the dominant species, while Borelli (2006) identified *Lb. plantarum* as the dominant species in this type of cheese. Perin et al. (2017) observed that LAB strains/species are influenced by geographical region, cheese farm and within each cheese farm throughout the year, with raw milk and "pingo" as important sources of bacterial diversity in the final product. The variation in LAB strains is also a result of the lack of standardization in the procedures for milking and cheese manufacturing among artisanal cheese farms.

Although the identification of 4 strains as *E. faecalis* brings up some concerns about their application in foods (*i.e.*, poor hygiene conditions, virulence factors, antibiotic resistance), it should be highlighted that this genus is naturally/commonly present in dairy products. In addition, these bacteria may have positive effects on cheese properties (Rivas et al., 2012). The presence of *Enterococcus* spp. in Brazilian artisanal cheeses is quite common as described by Guedes Neto et al. (2005), Lima et al. (2009) and Resende et al. (2011). The presence of enterococci in these products can be explained by their capacity to grow in diverse substrates and conditions, and to survive to pasteurization regimes normally employed in dairy products (Foulquié-Moreno et al., 2006). Enterococci are known to be significant members of the microbiota of fermented food, particularly in cheeses, contributing to ripening and organoleptic properties, and proteolytic activities. Enterococci can also produce bacteriocins, and are good candidates for acting as protective cultures, especially against *L. monocytogenes*. Stackebrandt and Teuber (1988) studied the molecular taxonomy and phylogenetic position of LAB and observed that DNA G + C content of *E. faecalis* and *L. monocytogenes* is quite close, resulting in a phylogenetic relationship of enterococci and listeriae, which explains, at least on part the ability of *Enterococcus* species to act as good anti-listerial agents. Prior research has clearly shown the capability of *Enterococcus* isolated from cheese to control *L. monocytogenes* (Ghrairi et al., 2004; Ribeiro et al., 2014; Rivas et al., 2012).

Although the growth potential of *L. monocytogenes* and cheese pH were significantly reduced with addition of anti-listerial LAB to cheeses produced with raw and pasteurized milk, the same did not happen with *L. monocytogenes* growth rates. A greater drop in pH was expected for treatments with LAB addition since the chosen strains purposely showed high acidifying capacity. According to Le Marc et al. (2002), at low temperatures the pH range at which *Listeria* can grow is narrower, and the combination of low temperature and pH should result in a reduced growth rate. Le Marc et al. (2002) noted that growth rates of *Listeria innocua* were reduced to zero in pH < 5.0 at different concentrations of lactic acid. However, in our study, *L. monocytogenes* growth rates appear to be less affected by pH decline due to its capacity of growing/surviving in a wide range of pH (4.0–9.5) (Melo et al., 2015). This may be explained by the observation of O'Driscoll et al. (1996) that some strains of *L. monocytogenes* have a significant adaptive acid tolerance response after exposure to mild acid conditions (pH 5.5).

The search for LAB with anti-listerial properties is important to control *L. monocytogenes* in Brazilian soft cheeses. While there are some studies that have analyzed the capability of LAB strains in inhibiting *L. monocytogenes* growth in Minas soft cheese, none have used LAB isolated from Minas artisanal cheeses. Pingitore et al. (2012) showed a bacteriostatic effect of bacteriocinogenic *Enterococcus mundtii* CRL35 against *L. monocytogenes* present in cheeses

stored for up to 12 days at 8 °C. On the other hand, bacteriocinogenic *Enterococcus faecium* ST88Ch and non-bacteriocinogenic *E. faecalis* ATCC 19443 were less effective in inhibiting *L. monocytogenes*, as the bacteriostatic effect occurred only after the 6th day. Nascimento et al. (2008) concluded that there was no significant difference in *L. monocytogenes* Scott A counts between the control cheese with starter culture only and those added of adjunct bacteriocinogenic cultures during 21 days of shelf-life. Naldini et al. (2009) concluded that the non-bacteriocinogenic starter culture was able to keep *L. monocytogenes* Scott A concentration at around 4 log CFU/g during 25 days at 5 and 10 °C. The above studies, as well as our results, suggest that LAB exert only a bacteriostatic effect in *L. monocytogenes* in Minas frescal cheese. In spite of this, the addition of LAB presenting anti-listerial activity (even if bacteriostatic) seems to comprehend a feasible strategy to contribute with the safety of Minas soft cheese. This strategy is supported by the findings that soft cheeses without addition of selected LAB consistently presented pronounced *L. monocytogenes* growth.

To the best of our knowledge, this is the first study that evaluated the fate of intentionally inoculated *L. monocytogenes* and indigenous mix of LAB in Minas-style semi-hard cheese during ripening. *L. monocytogenes* was only able to grow in cheeses containing natural microbiota. In addition, *L. monocytogenes* growth potential was significantly higher in cheese produced with pasteurized milk, which was expected since pasteurization eliminates a large part of background microbiota present in milk which may facilitates pathogen's growth. *L. monocytogenes* was inactivated in cheeses containing added LAB with known anti-listerial properties. The inactivation of this pathogen was more pronounced in cheese made with raw milk cheese. Furthermore, a significantly higher inactivation rate of *L. monocytogenes* was found in cheeses containing added LAB with known anti-listerial properties compared to cheese with no added LAB. While the final pH of cheeses did not differ significantly among all treatments, a higher drop in pH in the first 72 h of ripening was observed in cheeses added with selected LAB strains. The pH of these cheeses reached 4.66 within this period (Fig. 3), which may have contributed to the inactivation of *L. monocytogenes*. Cheeses without added LAB showed *L. monocytogenes* growth, and stationary phase was achieved before 72 h, while the pH of raw or pasteurized cheeses was still above 5.60. Wemmenhove et al. (2018) reported that the presence of undissociated lactic acid is the key reason for inhibition of *L. monocytogenes* growth in Gouda cheese. Besides, these authors indicated that low water activity, as presented after prolonged ripening times, can also cause growth inhibition. Here, it is hypothesized that the association of pH reduction and production of antimicrobial compounds may have resulted in *L. monocytogenes* inactivation in cheeses added of LAB.

Numerous studies have analyzed the fate of *L. monocytogenes* in different types of semi-hard cheeses, as affected by the addition of LAB with anti-listerial activity (Arqués et al., 2005; Langa et al., 2018; Millet et al., 2006; Rodríguez et al., 2001; Scatassa et al., 2017). Scatassa et al. (2017) showed that *L. monocytogenes* counts decreased by roughly 3 log CFU after 15 days in Pecorino Siciliano cheese produced with a mix of 3 anti-listerial LAB strains (*Lactococcus lactis*, *Lb. rhamnosus* and *E. faecium*); while in Vastedda della Valle del Belìce cheeses, the multi-strain LAB mixture prevented the growth of *L.* monocytogenes. There is only one Brazilian study (Pinto et al., 2009) that investigated the survival of *L. innocua* during ripening of cheese produced in the region of Serro. The cheese was produced from raw milk and "pingo" (natural starter), following ripening during 60 days at 30 °C. *L. innocua* survival was affected by time and inoculation level (10^3, 10^2 and 10^1 CFU/mL) and counts of ~2 log CFU/g was still detected in the Serro cheeses after 60 days of ripening at 30 °C.

It is known that Minas artisanal cheeses are typically ripened for 17–22 days depending on the region. This is in conflict with Brazilian federal guidelines which states that raw milk cheeses must be ripened for at least 60 days (MAPA, 2000). In an effort to reduce illegal sale of these cheeses, new legislation was passed in 2011 which allowed a reduced ripening time provided that specific studies could show the reduced time ensured the production of safe cheeses (MAPA, 2011). This new legislation still hampers the lives of small cheese producers who do not have the financial means to commission such studies. Several Brazilian research labs have investigated shortened ripening times to help these farmers meet the new standards. For instance, Dores (2007) observed that 22 days at ambient temperature (25 °C) was required to achieve the microbiological standards for Canastra cheese, while 35 days at refrigeration temperature (10 °C) was required to meet the same standard. Martins et al. (2015) showed that legislation standards were met after 17 days of ripening at room temperature for Minas artisanal cheese produced in Serro region. These same researchers showed that 33 days at (8 °C) were needed in the dry season and that time increased to 63 days at (8 °C) in the rainy season. All these studies show the challenges in designing a "one size fits all" Brazilian legislation, since ripening times vary greatly with regional styles, temperatures and seasons of the year. These studies also point out the clear benefits of room temperature aging in managing microbiological hazards during reduced ripening times. Despite this, it is important to highlight that these studies did not add pathogenic microorganisms to cheeses, but rather took samples directly from farmers and analyzed the physicochemical and microbiological parameters during ripening. The main limitation of this approach regards the statistical validity of samples taken, which may not ensure the adequate number of samples was collected to allow detection/enumeration of the microbial target. In our study, a high concentration of *L. monocytogenes* was intentionally added in the cheeses to verify if a mix of indigenous LAB with anti-listerial activity would be able to inactivate the pathogen or inhibit its growth during a short ripening time. Our results demonstrated that *L. monocytogenes* counts were reduced by 4 log CFU/g after about 15 and 21 days in semi-hard cheeses containing added LAB and produced with raw and pasteurized milk, respectively. Furthermore, our study shows that after 22 days at 22 °C, *L. monocytogenes* counts were reduced by 5.8 log CFU/g in raw milk semi-hard cheese. Cheeses produced with pasteurized milk needed a longer ripening period to achieve the same level of pathogen reduction likely due to the added benefit of indigenous LAB naturally present in raw milk. Raw milk is an important source of bacterial diversity present in the final product, which acts along with the LAB from "pingo" (natural starter) to aid in the control of *L. monocytogenes*.

5. Conclusion

Consumers' interest in safer traditional raw-milk cheeses with high organoleptic characteristics is increasing, and microorganisms play a major intrinsic role in this process. LAB strains with anti-listerial activity present great possibility for use in fermented dairy products as starter or adjunct cultures. In this study, six LAB strains [*Lactobacillus brevis* 2-392, *Lactobacillus plantarum* 1-399 and 4 *Enterococcus faecalis* (1-37, 2-49, 2-388 and 1-400)] with anti-listerial activity at 37 and 7 °C were isolated. Their further addition to artisanal Minas cheeses (Canastra-type) made with raw or pasteurized milk resulted in bacteriostatic effects and inactivation of *L. monocytogenes* in soft cheese and in semi-hard cheese, respectively. The inactivation of *L. monocytogenes* was significantly higher in semi-hard cheeses made with raw milk and added of

selected indigenous LAB. These findings suggest that the addition of these six LAB strains with anti-listerial activity may constitute an additional feasible hurdle to inhibit *L. monocytogenes* growth during storage of soft cheese and to inactivate this pathogen during the ripening period of semi-hard artisanal Canastra cheeses. Taking into account *L. monocytogenes* inactivation, the application of the indigenous LAB selected in this study would allow to complete the ripeness process of artisanal Canastra cheese at 22 °C within 22 days.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fm.2018.02.006.

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