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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.

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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

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