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Control of *Listeria monocytogenes* in fresh cheese using protective lactic acid bacteria

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ABSTRACT

In the past years, there has been a particular focus on the application of bacteriocins produced by lactic acid bacteria (LAB) in controlling the growth of pathogenic bacteria in foods. The aim of this study was to select LAB strains with antimicrobial activity, previously isolated from a traditional Azorean artisanal cheese (Pico cheese), in order to identify those with the greatest potential in reducing *Listeria monocytogenes* in fresh cheese. Eight bacteriocin producer strains identified as *Lactococcus lactis* (1) and *Enterococcus faecalis* (7) were tested. In general, the bacteriocin-producing strains presented a moderate growth in fresh cheese at refrigeration temperatures (4 °C), increasing one log count in three days. They exhibited slow acidification capacity, despite the increased production of lactic acid displayed by some strains after 24 h. Bacteriocin activity was only detected in the whey of fresh cheese inoculated with two *Enterococcus* strains, but all cheeses made with bacteriocin-producing strains inhibited *L. monocytogenes* growth in the agar diffusion bioassay. No significant differences were found in overall sensory evaluation made by a non-trained panel of 50–52 tasters using the isolates as adjunct culture in fresh cheese, with the exception of one *Enterococcus* strain. To test the effect of in situ bacteriocin production against *L. monocytogenes*, fresh cheese was made from pasteurized cows' milk inoculated with bacteriocin-producing LAB and artificially contaminated with approximately 10^6 CFU/mL of *L. monocytogenes*. The numbers of *L. monocytogenes* were monitored during storage of fresh cheese at refrigeration temperature (4 °C) for up to 15 days. All strains controlled the growth of *L. monocytogenes*, although some *Enterococcus* were more effective in reducing the pathogen counts. After 7 days, this reduction was of approximately 4 log units compared to the positive control. In comparison, an increase of 4 log CFU/mL in pathogen numbers was detected over the same period, in the absence of bacteriocin-producing LAB. The combination of two bacteriocin producing *Enterococcus* sp. optimized the reduction of *L. monocytogenes* counts in fresh cheese, reducing by approximately 5 log units after 7 days. The present work demonstrates that using bacteriocin-producing strains in the manufacture of fresh cheese might contribute to preventing the growth of undesirable pathogenic bacteria such as *L. monocytogenes*. A blend of two strains demonstrated great potential as a protective culture for the cheese making process.

1. Introduction

Listeria monocytogenes is the causative agent of listeriosis, a severe disease in humans and one of the most significant foodborne diseases in industrialized countries (Schlech, 2000). *L. monocytogenes* is a psychrotrophic microorganism widely distributed in the environment that can grow at refrigerated temperatures and is highly acidic and salt-tolerant (review by McLauchlin et al., 2004). These characteristics make *L. monocytogenes* difficult to control in dairy foods and in particular in soft cheeses. As a result, contaminated cheese has been implicated in some of the major listeriosis outbreaks reported worldwide (Almeida et al., 2013; Castro et al., 2012; Farber and Peterkin, 1991; Hitchins, 1996).

Latin-style fresh cheese is a popular dairy product in Portugal and Spain (Evert-Arriagada et al., 2013). It is a non-ripened cheese, produced by enzymatic coagulation of milk with rennet, without adding starter cultures. It exhibits a soft texture, a slightly acidic flavor and has low salt and high moisture content. Due to the absence of starters, fresh cheese presents high pH values (above 5.0) and should be consumed shortly after production. Fresh cheese is also typically made without preservatives, requiring refrigerated temperatures for conservation. Therefore, fresh cheeses are particularly sensitive to colonization by *L. monocytogenes* through post-process contamination (Kabuki et al., 2004). Due to the relatively high pH and water activity allowing the growth of this microorganism during cheese storage at 4 °C, fresh cheese deserves particular attention from a hygienic/safety perspective (Castro et al., 2012). Consequently, the use of additional strategies to control the growth and survival of *L. monocytogenes* is imperative.

One of the approaches used to prevent the growth of undesirable microorganisms in food is the use of bacteriocins or bacteriocin-

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producing lactic acid bacteria (LAB). Bacteriocins provide a more natural means of preserving food by reducing the requirement for chemical preservatives. A large number of bacteriocins possess anti-listerial activity and many of them have been applied to the control of *L. monocytogenes* in cheeses (Dal Bello et al., 2012; Liu et al., 2008; Ross et al., 1999).

Although underutilized in the majority of cases, some enterocins produced by enterococci are among the most active bacteriocins in combating *L. monocytogenes* and may offer potential benefits in controlling this microorganism in foods. However, direct application of bacteriocins/enterocins may result in decrease or the complete loss of antimicrobial activity due to problems related to interaction with food components (Aasen et al., 2003; Chollet et al., 2008). Alternatively, the incorporation of live bacteriocin-producing strain(s), either through direct addition to the food or in an immobilized form on packaging, may present a potential benefit in controlling *L. monocytogenes* in dairy products.

In this study, eight LAB bacteriocin producers isolated from an artisanal cheese (Pico cheese), and identified as *Enterococcus faecalis* (7) and *Lactococcus lactis* (1) were tested. The growth and in situ bacteriocin production of these LAB strains were investigated during the manufacture and storage of fresh cheese. Moreover, the effect of adding these LAB, on the physicochemical parameters and sensory acceptance of fresh cheese was evaluated. In addition, these bacteriocin-producing strains were tested with regard to controlling *L. monocytogenes* growth during the manufacture and storage of fresh cheese. The capacity for several blends of two *Enterococcus* strains to control *L. monocytogenes* growth during the manufacture and storage of fresh cheese was also assessed. To our knowledge, this represents the first report involving the use of bacteriocin producing LAB in fresh cheese with a view to controlling *L. monocytogenes*.

2. Material and methods

2.1. Microorganisms and culture conditions

A strain of *L. lactis* producer of lacticin 481 (L3A21M1) and 7 strains of *E. faecalis* previously isolated from an Azorean artisanal cheese (Ribeiro et al., 2014) were included in this study. Before each experiment, the cultures were incubated for 24 h at 30 °C in MRS broth (AES, Bruz, France). Prior to fresh cheese production, all revitalized strains were grown in ultra-pasteurized (UHT) skim milk and incubated for 48 h at 30 °C reaching a cell concentration of about 10^8 cells/mL. The indicator strain *L. monocytogenes* ATCC 7466 was propagated in Nutrient Broth (AES) and incubated for 18 h at 37 °C. For inoculation in cheese, the revitalized cells of *L. monocytogenes* were pelleted by centrifugation (Centrifuge 5415D, Eppendorf, Germany), for 10 min at 5900 *g*, washed twice and resuspended in buffered peptone water (AES) and subsequently diluted to give the desired cell number (10^8 CFU/mL).

2.2. Manufacture of fresh cheese

Cow's raw milk (3.5% fat, wt/wt) obtained from the Azores University farm (Chegalvorada, Angra do Heroismo, Portugal) was pasteurized at 73 °C for 16 s in a shaking water bath (Julabo, Model SW22, Germany) and then cooled in wet ice. Calcium chloride (0.2 g/L; Merck) and NaCl (10 g/L) were then added to the milk. In each trial, milk warmed at 32 °C was distributed in five 0.5 L vats and individually inoculated with each LAB culture (1%). Rennet (LMF 1/15,000, 0.2 g/L) was then added to the milk and incubated at 32 °C for aprox. 40 min. Control cheese was made without any inoculum. Once the coagulum was sufficiently firm, it was cut into 1–2 cm cubes and heated at 37 °C for 25 min. Whey was drained off and curds were distributed into perforated sterile stain steel circular cheese containers (6.5 cm in diameter). Cheeses were stored under refrigeration (4 °C) inside appropriated plastic boxes with a mesh covered with sterilized cheese cloth allowing whey drainage.

Cheeses were made in three trials carried out on six different days. In each day, five types of cheeses were made: one control (2 cheeses) and four inoculated with four different LAB strains (2 cheeses for each LAB).

2.3. Analysis of the experimental fresh cheeses

Cheeses were sampled in duplicate (two different cheeses of the same trial) for pH, titratable acidity, LAB counts and bacteriocin activity in the beginning of storage (time = 0) and after 0, 6, 24, 48 and 72 h of storage at 4 °C. Cheese pH was measured directly with a pH meter (WTW Inolab pH Level 1, Germany). Titratable acidity was determined by direct titration of 4 g of fresh cheese dissolved in warm water, according to AOAC method # 920.124 (AOAC, 1995).

For microbiological analyses, 25 g of cheese was transferred into a stomacher (400 Circulator, Seward, United Kingdom) containing 225 mL of sterile 0.1% (wt/vol) buffered peptone water (AES). Further dilutions were made from this original dilution and the quantification of microbial counts was carried out using the pour plate technique. The lactococci were enumerated on M17 agar (Biokar) and incubated under aerobic conditions at 30 °C for 72 h, whereas the enterococci were enumerated on KF Streptococcus agar (Biokar) at 37 °C for 48 h under aerobic conditions. Each experiment was conducted in duplicate.

The antimicrobial activity of cheese samples was detected by the agar disk diffusion assay (Ribeiro et al., 2014). Briefly, cheese samples (5 g) were centrifuged at 4500 *g* for 10 min. Supernatants were neutralized with phosphate buffer (0.5 M, pH 7.0), filtered through a 0.22 μm membrane filter (Sartorius Stedim Biotech, Germany) and placed in duplicate into wells (6 mm diameter) made in pour plates of Plate Count Agar (AES) containing cultures of *L. monocytogenes* as indicator microorganism. After anaerobic incubation at 37 °C for 12 h, the diameter of the zone of growth inhibition was measured and bacteriocin activity expressed in mm.

In addition, uniform cheese pieces were cut using a sterile cork borer (5 mm) and transferred into wells (6 mm diameter) of Plate Count Agar (AES) inoculated with indicator organism as previously indicated. The dishes were incubated at 37 °C for 12 h and checked for a clear halo around the cheese samples. All determinations were done using three independent samples (cheeses made on different days).

2.4. Sensory analysis

To evaluate the influence of the eight bacteriocin producers in the final sensorial characteristics of fresh cheese, the different cheese productions were subjected to a panel evaluation.

Sensory analysis was performed on fresh (2 days old) cheese by a non-trained panel of tasters comprising 50 to 52 participants from both genders, with ages ranging from 19 to 60 years old. The attributes judged were acidity, salty taste, firmness, flavor and general acceptability. Cheese scoring was conducted on a one to five scale (in which 1 stands for absence and 5 for presence at a strong level). Prior to assessment, each cheese was divided into various portions, and equilibrated at room temperature. Panelists were exposed to each sample on an individual petri plastic dish, and were asked to assess the specific attributes. Two evaluation sessions were performed and, in each session, four samples with LAB inoculate and one control (without any inoculums) were tested.

2.5. Evaluation of L. monocytogenes growth in fresh cheese

Prior to cheese production, all revitalized LAB isolates were grown in UHT skim milk and incubated for 48 h at 30 °C reaching a cell concentration of about 10^8 cells/mL. *L. monocytogenes* (1%) was added to the milk in a suspension of 10^5–10^7 CFU/mL, by the time of LAB inoculation (Section 2.2) and left for 20 min before rennet addition. Fresh cheeses were then prepared as indicated in Section 2.2. A control cheese was made containing only *L. monocytogenes* inoculate. To enumerate