



Effect of autochthonous bacteriocin-producing *Lactococcus lactis* on bacterial population dynamics and growth of halotolerant bacteria in Brazilian charqui



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ABSTRACT

Charqui is a fermented, salted and sun-dried meat product, widely consumed in Brazil and exported to several countries. Growth of microorganisms in this product is unlikely due to reduced *Aw*, but halophilic and halotolerant bacteria may grow and cause spoilage. Charqui is a good source of lactic acid bacteria able to produce antimicrobial bacteriocins. In this study, an autochthonous bacteriocinogenic strain (*Lactococcus lactis* subsp. *lactis* 69), isolated from charqui, was added to the meat used for charqui manufacture and evaluated for its capability to prevent the growth of spoilage bacteria during storage up to 45 days. The influence of *L. lactis* 69 on the bacterial diversity during the manufacturing of the product was also studied, using denaturing gradient gel electrophoresis (DGGE). *L. lactis* 69 did not affect the counts and diversity of lactic acid bacteria during manufacturing and storage, but influenced negatively the populations of halotolerant microorganisms, reducing the spoilage potential. The majority of tested virulence genes was absent, evidencing the safety and potential technological application of this strain as an additional hurdle to inhibit undesirable microbial growth in this and similar fermented meat products.

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1. Introduction

Charqui is a traditional salted and dried meat product largely consumed in Brazil and exported to several countries. For production, deboned meat muscles are injected with a brine solution, added of coarse marine salt and piled up on concrete floors to let juices drain out. The piles are inverted every 24 h for several days, and in the final step, the salted meat pieces are exposed to the sun on rails until *Aw* decreases to 0.7–0.8. At night, the meat pieces are removed from the rails and piled up. The whole process can take up to 10 days. All steps are carried out at room temperature. The final product is shelf-stable and can be stored without refrigeration up to six months. The unique sensorial features of charqui are generated during fermentation carried out by autochthonous

microorganisms, mainly *Lactobacillus*, *Pediococcus*, *Micrococcus* and *Staphylococcus* (Pinto et al., 2002).

Due to the low *Aw*, the growth of microbial pathogens in charqui is very unlikely, but halophilic and halotolerant spoilage bacteria, coming from the salt used in the manufacture of the product, can grow (Youssef et al., 2007). The halotolerant *Halobacterium cutirubrum* causes changes in odor and appearance of slime and red spots on the product surface (Shimokomaki et al., 1998; Pinto et al., 2002).

Recently, Biscola et al. (2013) reported that charqui is a good source of bacteriocinogenic lactic acid bacteria (LAB) capable of inhibiting the growth of spoilage halotolerant bacteria. The objective of the present study was to add one of these bacteriocinogenic strains (*Lactococcus lactis* subsp. *lactis* 69) to charqui and evaluate its ability to prevent the growth of spoilage bacteria during storage up to 45 days and its influence on the bacterial diversity during the manufacturing of the product. This diversity was evaluated using denaturing gradient gel electrophoresis (DGGE), one of the most

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powerful tools to monitor the microbial dynamic changes during food fermentations (Ercolini, 2004; Coccolin et al., 2009; Tu et al., 2010), with the advantage of detecting non-culturable microorganisms (de Vuyst and Vancanneyt, 2007; Ruiz et al., 2012). The safety of the strain for potential application in food was also checked by investigation of virulence genes.

2. Material and methods

2.1. Preparation of inocula for experimental contamination

The potential application of *L. lactis* 69 to prevent charqui spoilage was evaluated with two pools of halotolerant bacteria previously isolated from the product presenting the characteristic red spoilage (Biscola et al., 2013). The halotolerant strains have been identified by amplification and sequencing of 16S rDNA Biscola et al. (2013). One pool contained nine strains of medium halotolerant bacteria, able to grow in medium with 3% salt, and consisted of five strains of *Staphylococcus xylosus*, two strains of *Staphylococcus pasteuri*, one strain of *Staphylococcus warneri* and one strain of *Staphylococcus* sp. The other pool contained nine strains of highly halotolerant bacteria, able to grow in medium with 10% salt, and consisted of four strains of *S. xylosus*, one strain of *Staphylococcus saprophyticus* and four strains of *Staphylococcus* sp. The medium halotolerant bacteria were grown individually in 5 mL of Tryptone Soya Broth (TSB) (Oxoid, Basingstoke, UK) containing 3% (w/v) NaCl (Synth, Diadema, Brazil) at 37 °C for 24 h, and highly halotolerant bacteria were grown individually in 5 mL of TSB containing 10% (w/v) NaCl at 37 °C for 24 h. The two pools were prepared mixing 5 mL of each individual culture, achieving a final population of 10^8 CFU/mL.

The bacteriocin-producing strain used in the study was a *Lactococcus lactis* subsp. *lactis* strain (*L. lactis* 69), isolated from charqui (Biscola et al., 2013). For application in the charqui manufacture, *L. lactis* 69 was grown in de Man, Rogosa and Sharp (MRS) broth (Oxoid, Basingstoke, UK) at 30 °C for 24 h, to a final population of 10^9 CFU/mL.

2.2. Charqui manufacture

Charqui was manufactured in the pilot plant of a meat industry located in São Paulo, Brazil, following as closely as possible the procedure described by Shimokomaki et al. (1998), using six pieces of *Vastus lateralis* (commonly known as knuckle). Each piece weighed approximately 5 kg and thickness varied from three to five cm. The raw meat pieces were injected with a 25% NaCl (w/v) brine solution at 20% (v/w) using an automatic injector and piled on a concrete floor, separated from each other by a 1 mm thick layer of coarse marine salt. After 24 h, the piles were inverted and restacked, so that the uppermost pieces were repositioned at the bottom of the new piles. At each repositioning, the salt layers between the meat pieces were renewed. This procedure was repeated every 24 h for five days. The meat pieces were then washed to remove the excess salt, transferred to stainless steel rails and let sun-dry for 3–5 days, until moisture reached 45% and *Aw* 0.7–0.75. At night, the meat pieces were removed from the rails, piled on a concrete floor and covered with a canvas. All processing steps occurred at room temperature. The final product was packed in plastic bags and stored at room temperature. Fig. 1 shows a simplified flowchart of charqui processing.

Two lots of charqui were manufactured: one **control lot**, added of the two pools of halotolerant microorganisms (approx. 10^5 CFU/mL) and one **test lot**, added also of the bacteriocin-producing *L. lactis* 69 culture (approx. 10^6 CFU/mL). The microbial cultures

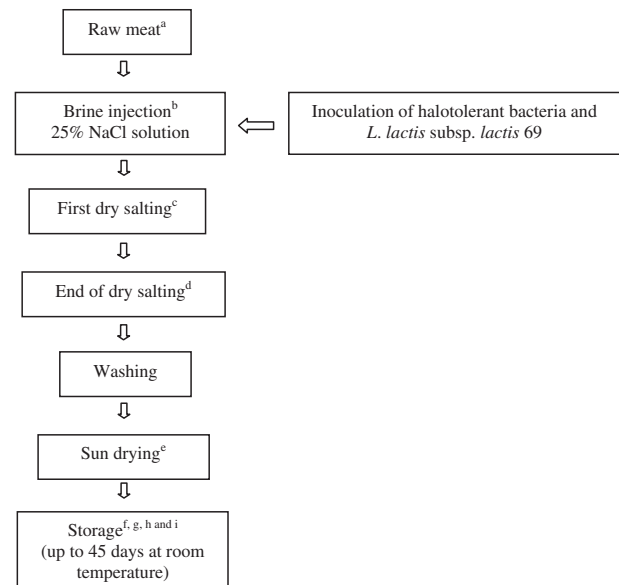


Fig. 1. Charqui processing flowchart (adapted from Shimokomaki et al., 1998), showing the sampling points (a to i) for microbiological testing and DGGE.

were added to the brine immediately before its injection in the raw meat pieces (45 mL of each pool in 45 L of brine).

The experiments were carried out as three independent replicates, i.e., each lot of charqui (control and test) was prepared three times, in different days.

2.3. Evaluation of the activity of *L. lactis* 69 against halotolerant bacteria in charqui

The activity of *L. lactis* 69 against halotolerant bacteria was evaluated in the meat in following steps of the manufacturing process (Fig. 1): after injection of brine, after dry salting, at the end of the dry salting steps, after sun drying, and after 5, 10, 25 and 45 days of storage. Enumeration of LAB in the product was performed in all these steps and also in the raw meat. At each sampling step, two analytical samples (duplicates) were withdrawn with a sterile knife and transferred separately to sterile plastic bags. Twenty-five grams of each analytical sample were transferred to a sterile plastic bag and stomached with 225 mL of sterile peptone water (0.1% w/v). The homogenates were subjected to serial decimal dilutions in sterile peptone water (0.1% w/v), and submitted to counts of halotolerant bacteria by plating in Tryptone Soya Agar (TSA) (Oxoid, Basingstoke, UK) containing 3% NaCl (w/v) and in TSA containing 10% NaCl (w/v) for enumeration of medium and highly halotolerant bacteria, respectively (Pinto et al., 2002). Enumeration of LAB was performed by plating on MRS Agar (Oxoid, Basingstoke, UK), as described by Hall et al. (2001). Results were expressed as log CFU/g and corresponded to the average of three independent experiments.

Results for test and control samples were compared using variance analysis and Tukey test (Statistica 7.0, 2004) and $p < 0.05$ as significance.

2.4. Evaluation of the effect of *L. lactis* 69 on the microbial diversity in charqui by DGGE

The microbial diversity present in the meat at the sampling points indicated in Fig. 1 was evaluated by denaturing gradient gel electrophoresis (DGGE) analysis focusing the 16S rDNA V3 region,

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