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Microbial ecology involved in the ripening of naturally fermented llama meat sausages. A focus on lactobacilli diversity

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ABSTRACT

Llama represents for the Andean regions a valid alternative to bovine and pork meat and thanks to the high proteins and low fat content; it can constitute a good product for the novel food market. In this study, culture-dependent and independent methods were applied to investigate the microbial ecology of naturally fermented llama sausages produced in Northwest Argentina. Two different production technologies of llama sausage were investigated: a pilot-plant scale (P) and an artisanal one (A). Results obtained by High-Throughput Sequencing (HTS) of 16S rRNA amplicons showed that the production technologies influenced the development of microbial communities with a different composition throughout the entire fermentation process. Both sequencing and microbiological counts demonstrated that Lactic Acid Bacteria (LAB) contributed largely to the dominant microbiota. When a total of 230 isolates were approached by RAPD-PCR, presumptive LAB strains from P production exhibited an initial variability in RAPD fingerprints switching to a single profile at the final of ripening, while A production revealed a more heterogeneous RAPD pattern during the whole fermentation process. The constant presence of *Lactobacillus sakei* along the fermentation in both productions was revealed by HTS and confirmed by species-specific PCR from isolated strains. The technological characterization of *Lb. sakei* isolates evidenced their ability to grow at 15 °C, pH 4.5 and 5% NaCl (95%). Most strains hydrolyzed myofibrillar and sarcoplasmic proteins. Bacteriocins encoding genes and antimicrobial resistance were found in 35% and 42.5% of the strains, respectively. An appropriate choice of a combination of autochthonous strains in a starter formulation is fundamental to improve and standardize llama sausages safety and quality.

1. Introduction

Fermentation and drying of meat are among the oldest technologies used to preserve foods. Fermented sausages are mainly produced using pork and/or bovine meat; however, in the Andean region of countries such as Argentina, Bolivia, Chile and Peru, llama (*Lama glama*) meat sausages are very popular. South America camelids have been domesticated 4000–5000 years ago by the Incas, and have been long used as a beast of burden, but also as a source of food and wool (Mamani-Linares et al., 2014). The ability to subsist in the extreme conditions of the bleak Andean plateau, together with the sharp increase in beef price, strongly influenced the increasing interest in llama rearing. Moreover, the importance of llama meat has increased recently due to its high protein nutritional value and reduced fat and cholesterol contents (Cristofanelli et al., 2004). Historically, llama meat products were restricted to dehydrated meat (jerky); however, an increased variety of different products started to be marketed mainly due to tourism development in the region. Among these, fermented Llama sausages are increasingly produced in Northwestern Argentina.

Ecological studies of this food product are of primary importance to understand the physical and chemical changes occurring during the fermentation and ripening process. The environmental determinants, as well as the manufacturing practice utilized, influence the establishment in the meat of a specific microbial consortium that will determine the rate of colonization (Bonomo et al., 2008). Studies on the microbiota composition of many traditional fermented sausages reveled the participation of Lactic Acid Bacteria (LAB), mostly *Lactobacillus sakei*, *Lactobacillus curvatus*, *Lactobacillus plantarum* and to a lesser extent species from *Pediococcus* and *Enterococcus* genera as was previously reported (Aymerich et al., 2003; Bonomo et al., 2008; Fontana et al., 2005, 2009, 2012). On the other hand, Gram-positive, catalase-positive cocci (GCC+), mostly *Staphylococcus* and *Kocuria* species and, less importantly, yeasts and molds are also involved in sausage fermentation. Among

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LAB, particularly *Lb. sakei* and *Lb. curvatus* have been widely documented to predominate during meat fermentation (Cocolin et al., 2009; Fontana et al., 2005, 2012; Pisacane et al., 2015; Rantsiou et al., 2006). LAB play a central role in meat fermentation and preservation process decreasing pH, producing bacteriocins to inhibit pathogens and contaminants, improving the sensory properties, the stability and the shelf-life of the final product (Fontana et al., 2005). The identification and technological characterization of LAB involved in meat fermentation are crucial to select the best-adapted strains to be used as functional starters (Rantsiou and Cocolin, 2006; Talon et al., 2007).

Developments in the field of molecular biology, allowed a number of methods to become available, which were applied to understand the dynamics and diversity of the microorganisms involved during sausages production (Cocolin et al., 2011). Moreover, cultures independent methods based on 16S rRNA gene amplification, together with High-Throughput Sequencing (HTS) technology have been recently applied with success to determine the bacterial communities present in food environments (Bassi et al., 2015; De Filippis et al., 2014; Połka et al., 2015; Stellato et al., 2015).

In order to design a starter culture for llama meat fermentation, relevant technological and safety characteristics of indigenous LAB should also be evaluated. The ability of the starter culture to compete with the autochthonous microbiota of raw materials and to undertake the metabolic activities expected, is conditioned by its growth rate and survival in the conditions prevailing during sausage fermentation (anaerobic atmosphere, high salt concentrations, low temperatures and low pH).

In this study, the microbial ecology of traditional llama fermented sausages from the Andean region of Argentina was investigated by HTS approach based on Illumina MiSeq sequencing of the V3-V4 16S rRNA and culture-dependent methods. The study of diversity and dynamics of LAB population as well as the technological and safety characterization of isolated strains were performed. The obtained data will be useful for the formulation of autochthonous starter cultures to be applied by the local meat industry in guided fermentations for the manufacture of higher quality and safer products.

2. Materials and methods

2.1. Sausages production and sampling procedures

Two independent productions of llama meat-fermented sausages were performed: one in a pilot-plant facility (P) from the Universidad Nacional de Jujuy (Laguna de los Pozuelos) and the other in an artisanal small factory (A) located in San Pablo, Jujuy (Northwestern Argentina). Both productions involved llama meat (75 and 71.5%) and the following ingredients: pork fat (22.5 and 25%), sodium chloride (2 and 2.4%), sugarcane (0.5 and 1.0%), black pepper (0.05 and 0.1%), crushed garlic (0.02 and 0.05) and nitrite/nitrate (0.02%) for production P and A, respectively. The meat batter was minced twice and stuffed into bovine casings. P production was fermented for 7 days at 24 °C and relative humidity (RH) of 95% followed by a gradual reduction of temperature to 15 °C and RH (from 92 to 89%) during the next 21 days. For A production, fermentation was carried out at 18 °C during 2 days and RH of 90%; a reduction in the temperature to 15–16 °C and RH to 85% was carried out during 18 days. The sampling was performed at 0, 1, 2, 4, 6, 10, 14 and 20 days for A production and at 0, 2, 4, 7, 14, 21, 28, and 35 days for P production; duplicate samples were collected and subjected to microbiological and molecular analyses for each production.

2.2. pH and a_w measurements

The pH potentiometric measurements were carried out with a pin electrode pH-meter (692 pH/Ion Meter-Metrohm, USA) that was inserted directly into the sample. The water activity (a_w) was determined with a water activity meter (AquaLab LITE, Decagon Devices, USA). For both, pH and a_w, three independent measurements were performed.

2.3. Microbiological analysis and preliminary physiological characterization of isolates

Ten grams of each sample were homogenized in 90 mL saline-peptone water (8.5 g/L NaCl, 1 g/L bacteriological peptone) using a Stomacher machine (Stomacher Lab-Blender 400, A.J. Seward Lab. London, UK) for 3 min. Decimal dilutions were then prepared and the following analysis were carried out: (i) total mesophiles (TM) bacteria on Plate Count Agar (48 h at 30 °C); (ii) LAB and lactobacilli on MRS and Rogosa agar media (Oxoid, Italy), respectively (48 h at 30 °C) under restricted oxygen conditions achieved using Anaerocult A (Merck, Germany); (iii) Gram-positive catalase-positive cocci (GCC) on mannitol salt agar (MSA) (48 h at 30 °C); (iv) total coliforms on McConkey agar (24 h at 37 °C); (v) enterococci on Slanetz and Bartley (SB) agar, (48 h at 37 °C); (vi) *Staphylococcus aureus* on Baird-Parker agar (Oxoid, UK) supplemented with egg yolk tellurite emulsion (Oxoid, UK) (24 to 48 h at 37 °C); (vii) *Listeria monocytogenes* on Palcam agar (Difco, Detroit, USA) and (viii) molds and yeasts on Rose Bengal Chloramphenicol agar (48 to 72 h at 30 °C). Cycloheximide solution (0.1%) was added to agar media to prevent yeast development. Unless otherwise specified, all media and ingredients were obtained from Oxoid (Basingstoke, UK). At each sampling time, five to ten colonies from MRS and Rogosa plates for each sample were randomly selected, transferred to MRS, incubated overnight at appropriate temperatures and stored at −20 °C in the same liquid media containing 20% glycerol before they were subjected to DNA extraction. All isolates were preliminarily characterized by means of cell morphology, Gram reaction and catalase activity.

2.4. DNA extraction, RAPD analysis and taxonomic identification of isolates

DNA extraction from isolates was performed using Microlysis (Labogen, UK) in accordance with the protocol described by the manufacturer and resulting DNA was used for the molecular identification of isolates. To achieve the strain fingerprints, isolates were subjected to PCR-RAPD analysis using primers RAPD2 (5′-AGC AGC GTC G-3′) and M13 (5′-GAG GGT GGC GGT TCT-3′) in separate reactions. The PCR conditions used for amplification experiments are those reported by Fontana et al. (2005). Amplification was performed in a GeneAmp PCR System 9600 thermocycler (Applied Biosystems). RAPD banding patterns were analyzed using Gel Compare software, Version 4.1. The Pearson correlation coefficient was used to calculate similarities in RAPD patterns; dendrograms were obtained by the unweighted pair group method with arithmetic averages. Taxonomical identification for Gram positive catalase negative isolates was achieved by species-specific PCR (sp-sp PCR) according to Berthier and Ehrlich (1999) and Quere et al. (1997) for *Lb. sakei* and *Lb. plantarum* identification, respectively. Sequencing of the 5′ region of 16S RNA gene according to Klijn et al. (1991) was also performed. The PCR mixture contained 1xMaster Mix PCR (Promega, Italy), 1.5 mM $MgCl_2$, 0.3 mM of each primer, and 2 μl of cell lysate as a template. Amplifications consisted of 1 cycle of denaturation for 5 min at 94 °C, 20 cycles at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and a final step at 72 °C for 7 min.

2.5. High-Throughput Sequencing (HTS) of 16S rRNA amplicons

Six sausage samples for P and A productions obtained at three different ripening time (T0, T14, T21 and T0, T10, T20 days, respectively) were selected for HTS approach. Total bacterial DNA was extracted from 200 mg of sausages for each sample using FastDNA® SPIN kit and Fast-Prep® Instrument (Qbiogene, Inc., CA) according to manufacturer instructions. Extracted DNA was examined on agarose gel and quantified using Quant-iT™ HS ds-DNA assay kit (Invitrogen, Paisley,