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Diversity and enzymatic profile of yeasts isolated from traditional llama meat sausages from north-western Andean region of Argentina

Lucía M. Mendoza [a,*], Beatriz Padilla [b], Carmela Belloch [b], Graciela Vignolo [a]

[a] Centro de Referencia para Lactobacilos (CERELA), Chacabuco 145, 4000 Tucumán, Argentina
[b] Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC), Avda. Agustín Escardino 7, 46980 Paterna, Valencia, Spain

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ABSTRACT

Llama meat fermented sausages are traditional products consumed in the Andean region of South America. The diversity and dynamics of yeasts present in these meat products were evaluated in two productions. The results demonstrated that yeast population increased during fermentation and it remained stable throughout the ripening period. A total of 414 yeasts isolated during different stages of production were identified and characterized by molecular methods. In both productions, *Debaryomyces hansenii* was found as the dominant species followed by *Candida zeylanoides*, although other species of the genera *Candida*, *Cryptococcus*, *Metschnikowia*, *Rhodotorula*, *Rhodosporium*, *Trichosporon* and *Yarrowia* also contributed to the fermentation. The fingerprinting analyses by RAPD-PCR of M13 minisatellite revealed the presence of different genotypes within *D. hansenii* and *C. zeylanoides* throughout the manufacturing process. Assay of proteolytic activity revealed that *Yarrowia lipolytica* from production A and *Candida deformans*, *Cryptococcus curvatus*, *Rhodotorula mucilaginosa* and *Rhodosporium diobovatum* from production B were able to hydrolyze meat proteins. All yeast species, excluding *Torulaspora delbrueckii*, exhibited lipolytic activity, whereas esterase activity was detected only in few species. A correspondence between enzymatic activity and RAPD M13 profiles was observed for yeasts of production A but this correspondence was not found in production B. An appropriate selection of yeast strains as starter cultures is fundamental for quality improvement of artisanal meat products.

1. Introduction

Fermented sausages are among the most important meat products elaborated worldwide. Sausages can be manufactured following artisanal or industrial procedures. In the artisanal process, meat is naturally fermented and drying of the product occurs over an extended time period. Nowadays, this procedure has been progressively replaced by industrial methods using starter cultures and controlled drying chambers to guarantee the safety and quality of the final products (Conventry & Hickey, 1991). Fermented sausages are mainly produced using pork meat (Andrade, Córdoba, Casado, Córdoba, & Rodríguez, 2010; Asefa et al., 2009; Cocolin, Urso, Rantsiou, Cantoni, & Comi, 2006); although, in the Andean region of countries such as Argentina, Bolivia, Chile and Peru, llama meat sausages are very popular. Meat from South American camelids, especially llama (*Lama glama*), is an important source of protein for the Andean population (Cristofanelli, Antonini, Torres, Polidori, & Renieri, 2005). Moreover, previous studies on the nutritional value of llama muscle have revealed that this meat offers some advantages due to its lower fat and cholesterol contents when compared to beef and pork meat (Coates & Ayerza, 2004; Polidori, Renieri, Antonini, Passamonti, & Pucciarelli, 2007).

During sausage fermentation, lactic acid bacteria and coagulase-negative cocci are by far responsible for physicochemical changes occurring in the meat (Fontana, Cocconcelli, & Vignolo, 2005; Martin et al., 2006). However, yeasts are also detected in high numbers during the production of fermented meat products, suggesting that these microorganisms could play an important role in sausage production (Cocolin et al., 2006; Mendonça, Gouvêa, Hungaro, Sodré, & Querol-Simon, 2013; Nuñez, Rodríguez, Córdoba, Bermudez, & Asensio, 1996). Several yeast species have been isolated from different dry-cured fermented sausages; literature findings support that the halotolerant species *Debaryomyces hansenii* constitutes the dominant and most frequently isolated yeast species (Asefa et al., 2009; Breuer & Harms, 2006; Encinas, Lopez-Diaz, Garcia-Lopez, Otero, & Moreno, 2000; Nuñez et al., 1996; Simoncini, Rotelli, Virgili, & Quintavalla, 2007). However, yeasts belonging to *Candida*, *Pichia*, *Rhodotorula*, *Hansenula* and *Cryptococcus* genera as well as *Yarrowia lipolytica* and *Metschnikowia pulcherrima* species have also been found during meat fermentations (Aquilanti et al., 2007; Cocolin et al., 2006; Encinas et al., 2000). The contribution of yeasts to flavor and texture development during the ripening of meat products has been previously investigated (Arboles & Julia, 1999; Miteva, Kirova, Gadjeva, & Radeva, 1986; Olesen & Stahnke,

* Corresponding author at: Chacabuco 145, 4000 Tucumán, Argentina. Tel.: +54 3814310465; fax: +54 3814005600.
E-mail address: lmendoza@cerela.org.ar (L.M. Mendoza).

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2000). Proteolysis and lipolysis are considered the most important enzymatic activities of yeasts contributing to the final characteristics of fermented meat products (Andrade, Córdoba, Sánchez, Casado, & Rodríguez, 2009; Durá, Flores, & Toldrá, 2004; Flores, Durá, Marco, & Toldrá, 2004; Patrignani et al., 2007).

The aim of this study was to evaluate the diversity and dynamics of yeasts isolated during different stages of the manufacturing process of traditional llama fermented sausages from the Andean region of Argentinian Northwest. Yeasts were identified and characterized using molecular methods and enzymatic characterization of selected strains was also performed.

2. Materials and methods

2.1. Llama sausage production and sampling

The fermented sausages sampled in this study were prepared using traditional techniques in small local meat factories in the province of Jujuy (Argentina). Production A (Laguna de los Pozuelos) and production B (San Pablo) were carried out without the use of starter cultures and the following ingredients were used: llama meat (70%), pork fat (27%), salt (2.5%), sugar (0.5%), sodium nitrite (250 ppm) and seasonings (black pepper, garlic, and red wine). The mixed ingredients were used to fill natural casings 10 cm long and 40 mm in diameter. Llama sausages were naturally fermented at 22–24 ºC and relative humidity (RH) 85–95% during 4 and 1 days for productions A and B, respectively. The fermentation step was followed by a ripening step which occurred for 24 days at 18–20 °C for production A while production B was carried out for 19 days at 13–15 °C. Samples were aseptically collected at 0, 2, 4, 7, 14, 21 and 28 days of processing for production A and at 0, 1, 2, 4, 6, 10, 15 and 20 days for production B. These productions were selected because both represent the typical methodologies used in the Andean region for production of artisanal llama sausages in the absence of a standardized industrial process.

Samples were maintained under refrigeration at 4 °C until analysis.

2.2. Physicochemical analysis

pH values were obtained by directly inserting the tip of the probe (Meat pHmeter, Hanna Instruments, Woonsocket, RI, USA) into the samples. Water activity (a_w) was determined on 5 mm sausage slices using an Aqua Lab instrument (Decagon Devices, Inc., Pullman, WA, USA). Three independent measurements were done on each sample.

2.3. Microbiological analysis

Ten grams of each sample containing both meat and casings were homogenized in 90 mL of sterile saline solution (0.9%) using a Stomacher 400 Lab Blender (Seward, UK). for 8 min at normal speed. Yeast counts were carried out in triplicate on Malt Extract Agar (malt extract 50 g/L, agar 20 g/L) after serial decimal dilution of the homogenized samples in saline solution. For yeast isolation, Malt Extract Agar plates and GPYA plates (glucose 20 g/L, peptone 5 g/L, yeast extract 5 g/L and agar 20 g/L) were supplemented with chloramphenicol (100 mg/L) and ampicillin (50 mg/L) to prevent bacterial growth. Plates were incubated at 28 °C up to 5 days. Approximately 20 colonies from Malt Extract Agar and 10 from GPYA were picked up randomly. Isolates were purified by streak plating and subcultured onto GPYA medium. The purified isolates were maintained in 15% (v/v) glycerol at −80 °C.

2.4. Molecular yeast identification

2.4.1. PCR-RFLP analysis of ITS1-5.8S rRNA-ITS2 region

Identification of the isolates was performed by PCR-RFLP analysis following protocols described by Esteve-Zarzoso, Belloch, Uruburu, and Querol (1999). Cells were directly collected from a fresh yeast colony and suspended in PCR reaction mix containing primers ITS1 (5-TCCGTAGGTGAACCTGCGG-3) and ITS4 (5-TCCTCCGCTTATTGATATGC-3) (White, Bruns, Lee, & Taylor, 1990). PCR reactions were performed in 50 μL final volume containing 5 μL of 10x buffer, 100 μM deoxynucleotides, 1 μM of each primer, 1.5 mM of $MgCl_2$, 1 unit of Taq polymerase (Invitrogen, Carlsbad, CA, USA) and DNA diluted to 10–20 ng/μL. Amplifications were carried out in a Bio-rad thermal cycler (MyCycler™, Bio-rad, Berkeley, California) with the following conditions: initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were digested without further purification with the restriction enzymes *Cfo*I, *Hae*III and *Hinf*I. Reaction mixtures contained 2 μL 10x digestion buffer, 7 μL deionized H_2O, 1 μL restriction enzyme and 10 μL PCR product. The mixtures were incubated overnight at 37 °C. PCR products and their restriction fragments were analyzed on 1% and 3% agarose gels, respectively, in 1x TAE buffer. Gels were stained with GelRed (Biotium, San Francisco, CA, USA). Fragment lengths were estimated using a 100 bp molecular weight marker (Invitrogen, Carlsbad, CA, USA). The obtained restriction profiles were compared with those recorded in the Yeast-id database (www.yeast-id.com).

2.4.2. Sequencing of the D1/D2 domain of the large subunit (26S) rRNA

Sequencing of the D1/D2 domain of 26S rRNA was carried out for the isolates that did not show a restriction profile available in database as well as for confirmation of the species assignations. NL1 (5-GCATATCAATAAGCGGAGGAAAAG-3) and NL4 (5-GGTCCGTGTTTC AAGACGG-3) primers (Kurtzman & Robnett, 1998) were used for the amplification. PCR reactions were performed under the same conditions used to amplify the ITS-5.8S rRNA region. Bands of PCR products were cut from the agarose gel and purified with a High Pure PCR Purification kit (Roche, Basel, Switzerland) following the manufacturer's instructions. Sequencing reactions were carried out in an automatic DNA sequencer (Applied Biosystems model 3130, California, USA). The sequences were assembled and analyzed using MEGA 5 software and subsequently compared with those in the GenBank using the BLASTN tool (NCBI).

2.5. RAPD-PCR analysis

The isolates from the most abundant yeast species were characterized using RAPD-PCR with M13 primer (5-GAGGGTGGCGGTTCT-3) as described by Huey and Hall (1989). DNA extraction was carried out from yeast cultures grown in GPY broth for 24 h at 28 °C (Querol, Barrio, Huerta, & Ramon, 1992). Reactions were performed in a final volume of 50 μL containing 5 μL of 10x buffer, 100 μM deoxynucleotides, 1 μM of primer, 1.5 mM of $MgCl_2$, 1 unit of Taq polymerase and 2 ng/μL of DNA. The amplification program was as follows: one cycle of 95 °C for 5 min, 40 repetitions of 93 °C for 45 s, 44 °C for 1 min and 72 °C for 1 min, and extension at 72 °C for 6 min. RAPD-PCR products were separated by electrophoresis on 2% agarose gels in 1x TAE buffer at 80 V.

2.6. Cluster analyses

Digitized images were analyzed with the Software BioNumerics version 6.6.4 using a trial license (Applied Maths, Kortrijk, Belgium). Normalization of band patterns was done using 100 pb molecular weight ladders (Invitrogen, Carlsbad, CA, USA) every tenth track. The levels of similarity between pairs were calculated using the Pearson correlation coefficient. Dendrograms for RAPD profiles were generated using the Unweighted Pair Group Method with Arithmetic mean algorithm (UPGMA) method.