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

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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.

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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,

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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.

Materials and methods

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.

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.

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.

Molecular yeast identification

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).

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).

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.

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.

Determination of main enzymatic activities

The ability of yeasts to secrete extracellular enzymes was evaluated by qualitative assays. Extracellular protease production was evaluated using myofibrillar and sarcoplasmic proteins obtained from beef (Fadda et al., 1999) and added at a final concentration of 1 mg/mL into a medium containing tryptone 5 g/L, yeast extract 2.5 g/L, glucose 1 g/L, and agar 15 g/L, pH 6.9. After incubation, the agar layers with meat proteins were removed from Petri dishes and stained for 5 min in 0.05% (w/v) Brilliant Blue R (Sigma, St. Louis, Missouri, USA) in methanol, acetic acid, and distilled water (50:10:40) according to protocols reported by Molina and Toldrá (1992). The presence of a clear zone surrounding the colony indicated proteolytic activity. Lipase activity was tested onto tributyrin agar medium (peptone 5 g/L, yeast extract 3 g/L, tributyrin 10 g/L and agar 15 g/L, pH 6.0) as described by Buzzini and Martini (2002). A clear halo around the colony indicated lipase activity.

The ability to hydrolyze fatty acid esters was tested on a medium containing peptone 10 g/L, NaCl 5 g/L, $CaCl_2 \cdot 2H_2O$ 0.1 g/L, Tween 80 (polyoxyethylene-sorbitan-monooleate) 10 g/L and agar 20 g/L, pH 6.8 (Slifkin, 2000). The esterase activity was detected as a visible precipitate (opaque halo) around the colony. For all enzymatic activities plates were incubated at 28 °C for 5 days.

Results

Physicochemical parameters

The results from pH and a_w measurements during the manufacturing of llama sausages are reported in Fig. 1. For production A, the values of pH decreased steadily from the initial 5.74 to 5.37 during the first 7 days (Fig. 1A) and subsequently it increased reaching a final value of about 5.67 at the end of ripening. a_w values decreased slowly during

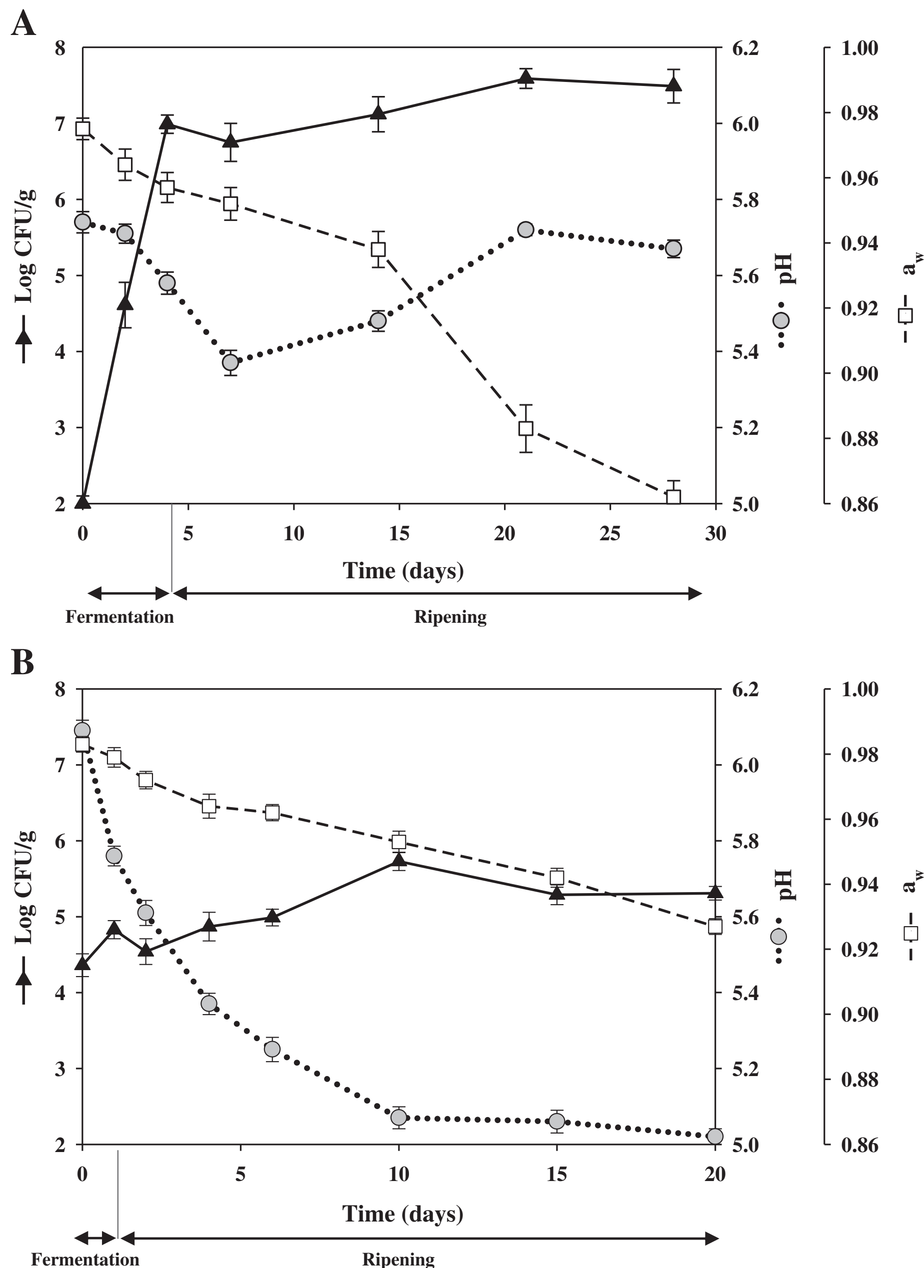

Fig. 1. Yeast counts (▲), pH (●) and a_w (□) trends during manufacturing process of llama fermented sausage for productions A (A) and B (B), respectively. Data are mean values of three experiments ± SD.

the first days of the process but a marked decrease was observed showing a value of approximately 0.86 towards the end. On the contrary, production B (Fig. 1B) showed an initial pH of about 6.07 which decreased progressively during the process reaching a final value of 5.02. a_w values showed a slow reduction throughout the manufacturing process reaching a final value of about 0.93.

Yeast counts, isolation and identification during manufacturing of llama sausages

Yeasts enumeration in Malt Extract Agar medium throughout the fermentation and ripening periods of llama sausages revealed differences between both productions. As shown in Fig. 1A, at the beginning of production A, a low yeast population (<2 log CFU/g) was detected although the total counts increased to 7 log CFU/g during the fermentation stage, and remained at that level until the end of the ripening process. In contrast, production B (Fig. 1B) was characterized by higher initial yeast counts of about 4 log CFU/g, with a slight increase during both fermentation and ripening stages, reaching the highest value (5.73 log CFU/g) at day 10.

A total of 414 yeast isolates were identified by PCR-RFLP analysis of the 5.8S-ITS rRNA region and sequencing of the D1/D2 domains of the 26S rRNA gene (Table 1). Homologies of 99–100% were obtained by comparison of the D1/D2 26S rRNA gene sequences of the yeast isolates with sequences of corresponding species in GenBank.

Five and thirteen different yeast species were identified, respectively, for productions A and B (Fig. 2A and B). Independently of the sausage production, *D. hansenii* and *Candida zeylanoides* were the main species present in all samples. In production A, these species represented 57.6% (*D. hansenii*) and 37.2% (*C. zeylanoides*) of total isolates. *D. hansenii* dominated from day 2 until day 7 of the manufacturing process whereas isolation frequency of *C. zeylanoides* increased at the last days of the ripening stage. The remaining species identified were *Cryptococcus kuetzingii*, *M. pulcherrima* and *Y. lipolytica*. *Cryp. kuetzingii* was only isolated in the sample taken at day zero whereas the other two species were found in low numbers at the middle of the ripening stage. Production B showed a more complex yeast species distribution. *D. hansenii* and *C. zeylanoides* represented 50.5 and 19.1% of isolates, respectively, while *Candida parapsilosis*, *Rhodotorula mucilaginosa*, *Cryptococcus curvatus* and *Cryp. kuetzingii* were also present in significant numbers during the first 6 days of the manufacturing process. Other yeasts within the *Cryptococcus*, *Trichosporon* and *Rhodotorula* genera were also detected in very low numbers until the middle of the ripening process.

Study of variability and distribution of genotypes

Population diversity within *D. hansenii* and *C. zeylanoides* was explored by genotyping of 149 isolates using the RAPD-PCR of M13. Diversity of M13 profiles was analyzed using UPGMA clustering methods (Fig. 3A and B). In particular for *D. hansenii* (Fig. 3A), ten clusters were distinguished at a similarity level of 65%. Cluster I grouped strains isolated from the middle of the manufacturing process for both sausage productions while cluster II, only contained isolates from production A obtained at 7, 21 and 28 days. Strains belonging to clusters III and IV were the most abundant during the fermentation stage in both productions. Isolates grouped in clusters V to IX were exclusively isolated from production B. Cluster X comprised strains present from fermentation to ripening in both productions. For *C. zeylanoides* (Fig. 3B), cluster analysis using a similarity coefficient of 65%, resulted in 7 clusters. Most of the isolates were included in clusters I, III and IV but two strains did not group and formed single-strain clusters. Cluster I grouped isolates of the last stage of manufacturing for production A as well as strains detected at the beginning of production B. Yeasts isolated from the first day of ripening in production B constituted cluster II. Cluster III contained isolates found at day zero in both productions and strains predominant in the last days of ripening for production B. Finally, yeasts isolated at the end of the process in production A constituted cluster IV. The remaining clusters were formed by isolates detected only in production B throughout the ripening stage.

Evaluation of main enzymatic activities of yeasts for sausage production

Results of qualitative evaluation for protease, lipase and esterase activities of yeasts isolated from llama sausages are shown in Table 2. Representative isolates from each species were selected for this screening.

Degradation of sarcoplasmic proteins was positive in species *Candida deformans*, *Cryp. curvatus*, *R. mucilaginosa*, *Rhodosporium diobovatum* and *Y. lipolytica* which were the only species exhibiting proteolytic activity against sarcoplasmic proteins while no activity was found when myofibrillar proteins were used as substrate (Table 2).

Lipase activity was detected in all sausage isolates with the exception of *Torulaspora delbrueckii*. Species *C. deformans*, *Rh. diobovatum*, *Trichosporon japonicum*, *Trichosporon montevideense* and *Y. lipolytica* as well as some *C. zeylanoides* and *D. hansenii* strains showed the highest lipase activity, whereas a moderate lipase production was determined for the remaining species and most of isolates of *C. zeylanoides* and *D. hansenii*.

Table 1
Identification by PCR-RFLP analysis and sequencing of the D1/D2 of 26S rDNA gene of yeasts isolated from llama fermented sausages.

Species	PCR (bp)	Restriction fragments (bp)			GenBank accession number[a]
		Cfo I	*Hae* III	*Hinf* I	
Candida deformans	380	210 + 170	380	180 + 100	HE660051
Candida parapsilosis	550	300 + 240	420 + 110	280 + 260	JX441605
Candida pararugosa	420	270 + 180	420	220 + 200	GU904205
Candida zeylanoides	630	295 + 295	420 + 140 + 80	315 + 315	KF273865
Cryptococcus curvatus	500	270	500	300 + 240	HQ323253
Cryptococcus kuetzingii	630	330 + 300	500 + 70 + 60	350 + 280	AF181546
Debaryomyces hansenii	650	300 + 300 + 50	420 + 150 + 90	325 + 325	KF273863
Metschnikowia pulcherrima	400	205 + 100 + 95	280 + 90	200 + 190	KC006526
Rhodosporidium diobovatum	650	300 + 220 + 110	550	230 + 120	KC494745
Rhodotorula mucilaginosa	620	300 + 220 + 100	410 + 200	340 + 200	KC442281
Rhodotorula slooffiae	600	570	600	320 + 250	KF303792
Trichosporon japonicum	550	280	500	230 + 80	EU882091
Trichosporon montevideense	550	280	500	220 + 150 + 100 + 80	JN939467
Torulaspora delbrueckii	800	330 + 220 + 150 + 100	800	410 + 380	KF268245
Yarrowia lipolytica	380	210 + 170	380	190 + 190	KC544463

[a] The D1/D2 26S rDNA gene sequences determined in this study showed 99–100% sequence similarity with the GenBank ACCN numbers listed.

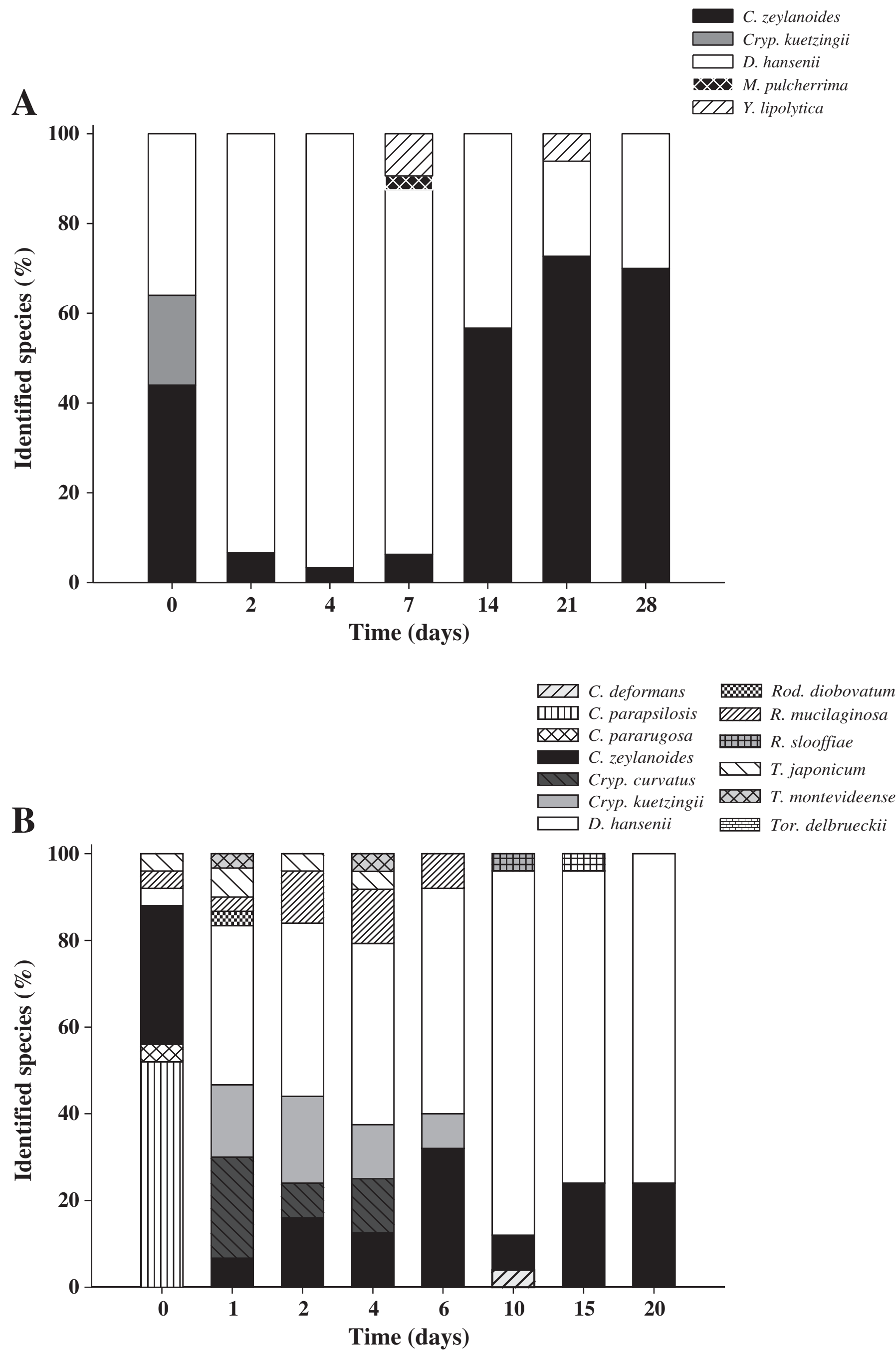

Fig. 2. Distribution of yeast species throughout the llama sausage elaboration for productions A (A) and B (B), respectively.

Concerning esterase, this activity was found to be strain dependent. A high percentage of isolated yeasts showed weak or negative esterase activity except for species within *Cryptococcus* genus as well as *Rh. diobovatum*, *T. japonicum* and some *C. zeylanoides* strains.

In addition, a correspondence between enzymatic activity and M13 profiles was noticed for yeasts from production A. Strains pertaining to *D. hansenii* and *C. zeylanoides* isolated during 14 to 28 days of process and corresponding to clusters II (Fig. 3A) and IV (Fig. 3B), respectively, showed higher lipase and esterase activities than those strains isolated from an early stage of sausage production. For yeasts belonging to production B, enzymatic activities were shown to be species-dependent and no variations were observed among different isolates throughout the manufacturing process.

Discussion

Fermented sausages are traditional meat products characterized by a great diversity in production methods and organoleptic traits among different countries and regions of the same country (Rantsiou et al., 2005). The literature has extensively reported studies about the microbiota and characteristics of different dry-cured products, particularly sausages produced from pork meat (Andrade et al., 2010; Cocolin et al., 2006). In the Andean region of South America, llama meat is a source of high nutritional value; moreover, it also represents the most available meat source in this region due to the impairment of the production of other livestock because of the extreme environmental conditions of the area. In addition, attention has recently been paid to llama meat products due to their lower

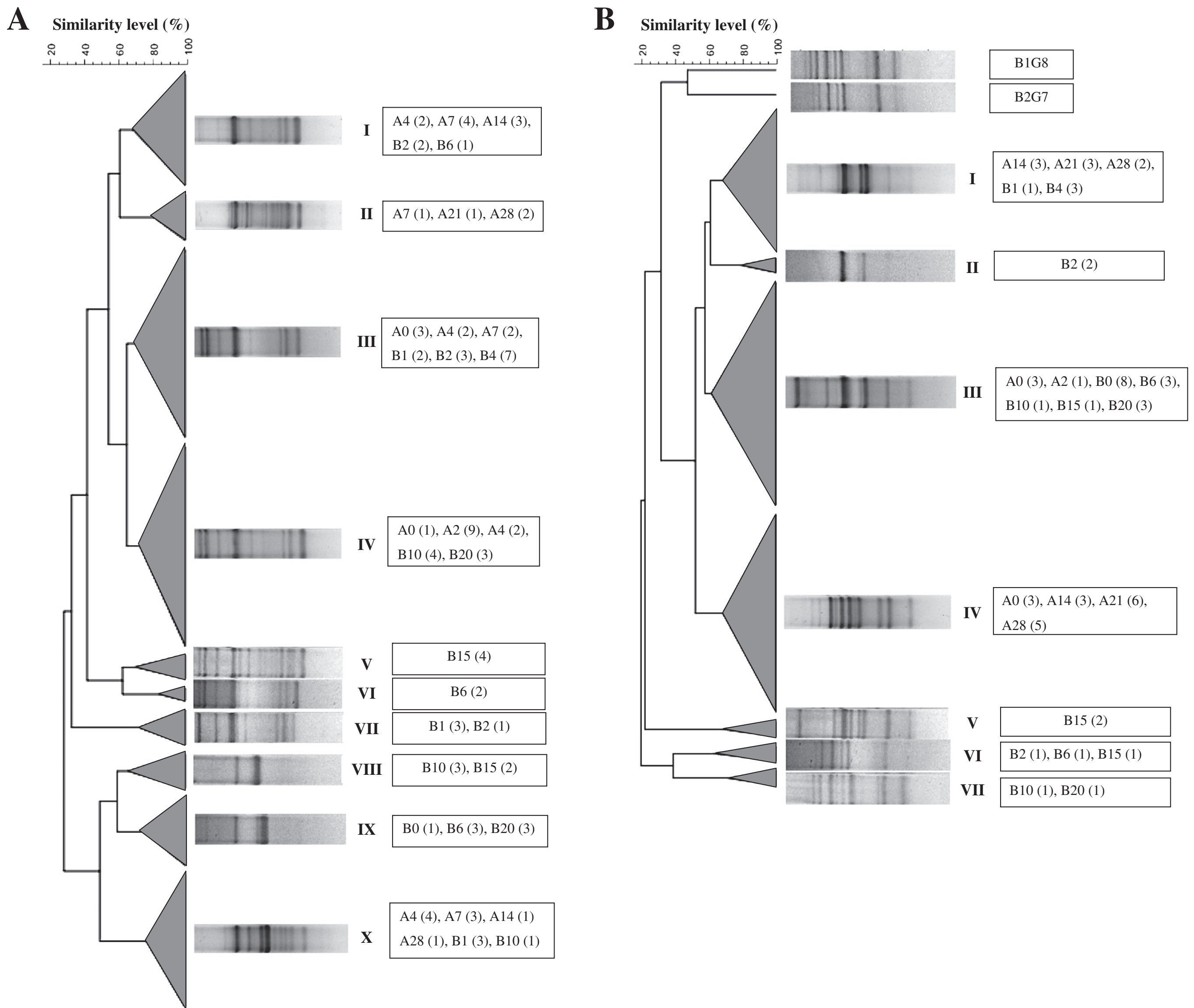

Fig. 3. Dendrograms generated from UPGMA cluster analysis based on Pearson correlation coefficient for normalized RAPD-M13 profiles from strains of *D. hansenii* (A) and *C. zeylanoides* (B). Strips show a representative profile for each cluster. The scale indicates the similarity percentage. Box code meaning: production, sampling day of sampling and number of isolates.

fat (12.6%) and cholesterol (52.8 mg/100 g) contents than beef and pork meat (Coates & Ayerza, 2004; Polidori et al., 2007).

In this study, the isolation and identification of yeast population during llama sausage manufacturing was carried out. Physicochemical characterization showed a typical evolution of pH and a_w throughout fermentation and ripening periods, being in agreement with those previously reported (Cocolin et al., 2006; Fontana et al., 2005; Mendonça et al., 2013; Patrignani et al., 2007). The pH decreased during fermentation but it increased in the ripening stage for sausages of production A. This increase in the pH may be due to the formation of N non-protein compounds and basic ammonium ions coupled with buffering actions of protein (Astiasaran, Villanueva, & Bello, 1990; Patrignani et al., 2007). Microbial activity has an important role on pH variation on the sausage production process. Lactic acid bacteria decrease the pH at the beginning of the ripening process, while yeasts increase this value as some of them have been reported as deacidifying microorganisms able to consume organic acids (Bonaïti, Leclercq-Perlat, Latrille, & Corrieu, 2004). For production B, pH values decreased throughout all manufacturing process. This different behavior could be due to a lower yeast population with respect to production A as well as higher lactic acid bacteria counts (data not shown) found in these llama sausages. On the other hand, a_w decreased from the beginning to the end of the process, although was higher during production A. Temperature could be other factor responsible for differences found between both llama meat fermentations. The ripening process for both productions was carried out at temperatures with 5 °C of difference which can influence microbial growth and metabolism.

Regarding yeast counts, significant differences were observed between productions; nevertheless, results are in accordance with other works on similar fermented meat products (Aquilanti et al., 2007; Cocolin et al., 2006). The dominant species in both productions of llama sausages was *D. hansenii* followed by *C. zeylanoides*. It has been well documented that *D. hansenii* is the main species isolated from different dry-cured meat products (Andrade et al., 2010; Asefa et al., 2009; Mendonça et al., 2013). This species is considered an extremophilic organism because of its halotolerance and ability to grow in low a_w habitats (Breuer & Harms, 2006). *C. zeylanoides* has been previously reported as the second most frequently isolated yeast species from meat products. This yeast species seems to dominate at the early stage of dry-cured meat production while its numbers decrease at the end of

Table 2
Qualitative evaluation of enzymatic activities of yeasts isolated from llama sausage.

Yeast species	Enzymatic activities[a]		
	Esterase	Lipase	Protease
Candida deformans	−	++	++
Candida parapsilosis	−	**w**	−
Candida pararugosa	−	**w**	−
Candida zeylanoides[b]	+/++	+/++	−
Cryptococcus curvatus	+	++	+
Cryptococcus kuetzingii	+	+	−
Debaryomyces hansenii[b]	**w**/+	+/++	−
Metschnikowia pulcherrima	**w**	**w**	−
Rhodosporidium diobovatum	+	++	+
Rhodotorula mucilaginosa	**w**	+	+
Rhodotorula slooffiae	−	+	−
Trichosporon japonicum	+	++	−
Trichosporon montevideense	−	++	−
Torulaspora delbrueckii	−	−	−
Yarrowia lipolytica	**w**	++	++

[a] Activity was determined by measuring the diameter of halo: −, no activity; **w**, weak activity (1–2 mm); +, moderate activity (2–3 mm); ++, strong activity (>3 mm).
[b] These species showed different levels of esterase and lipase activities.

ripening (Nielsen, Jacobsen, Jespersen, Koch, & Arneborg, 2008; Simoncini et al., 2007). However, we found *C. zeylanoides* in high numbers at day zero and also at the end of the ripening stage in production A. Moreover, *C. zeylanoides* was present during production B in moderate numbers along the whole sausage production process. These differences in the distribution of *C. zeylanoides* are probably due to variations in the production conditions such as temperature and RH used during sausage elaboration. Regarding the species of *Candida*, *Cryptococcus*, *Metschnikowia*, *Rhodotorula*, *Torulaspora* and *Yarrowia* genera, less often isolated during llama sausage processing, most of them have already been reported in fermented sausage manufacturing (Aquilanti et al., 2007; Encinas et al., 2000). Some species of *Trichosporon* genus were found in other types of fermented sausages (Coppola, Mauriello, Aponte, Moschetti, & Villani, 2000; Viljoen, Dykes, Callis, & von Holy, 1993). However, *T. japonicum* and *T. montevideense* have been reported for the first time in fermented sausages; suggesting that these yeast species could be related to the utilization of llama meat.

The presence of different strains of *D. hansenii* and *C. zeylanoides* in llama sausages was revealed by RAPD analysis as proposed by other authors (Andrighetto, Psomas, Tzanetakis, Suzzi, & Lombardi, 2000). Reproducibility problems of the RAPD technique were avoided using standard and rigorous protocols as well as internal controls of few strains which were included in all PCR DNA amplifications (Ramos et al., 2008).

Ten and seven M13 profiles were found for *D. hansenii* and *C. zeylanoides*, respectively. The diversity of genotypes within both yeast species isolated from llama sausages is in agreement with previous studies of other authors on fermented pork meat sausages (Andrade et al., 2010; Cano-García, Flores, & Belloch, 2013). The succession of different genotypes of *D. hansenii* and *C. zeylanoides* observed during the production of llama sausages was also in agreement with studies by Cocolin et al. (2006) who reported that a shift in the *D. hansenii* population took place during the manufacturing process of naturally fermented Italian sausage.

The evaluation of the enzymatic activities showed differences not only between species but also within isolates from the same species. Proteolysis is one of the most important biochemical changes during the ripening of fermented sausages which influences both texture and flavor development through peptide and amino acid formation (Hughes et al., 2002). Several works (Durá et al., 2004; Van den Tempel & Jakobsen, 2000) have reported that *D. hansenii* showed null or scarce contribution to fermented sausage proteolysis while other authors found important levels of activity in some strains of this species (Patrignani et al., 2007) suggesting that this enzymatic property is strain dependent for *D. hansenii*. In our study *D. hansenii* strains isolated from llama sausages did not show proteolytic activity.

In regard to lipases and esterases, these enzymes are gaining interest in the food industry for its contribution to flavor development (Bankar, Kumar, & Zinjarde, 2009). Some *D. hansenii* strains, in particular those isolated during the ripening stage showed the highest lipolytic activity levels, whereas esterase activity was scarcely found for this yeast species.

On the other hand, *C. zeylanoides* has been previously associated with various meat products and it has been characterized by its lipolytic activity and ability to grow at refrigeration temperatures (Nielsen et al., 2008; Nuñez et al., 1996). *C. zeylanoides* strains isolated from llama sausages exhibited lipolytic and esterase activities. Remarkably, our results showed a relationship between some *C. zeylanoides* and *D. hansenii* genotypes and enzymatic activities in production A, as strains from both species isolated from the last stage of the ripening process showed the highest lipase and esterase activities. Regarding the other llama sausage yeast species, it is noteworthy that *C. deformans*, *Cryp. curvatus*, *R. mucilaginosa*, *Rh. diobovatum* and *Y. lipolytica* were able to hydrolyze meat proteins. Among lipolytic species, high activity for *Y. lipolytica*, *C. deformans*, *Rh. diobovatum* and *Trichosporon* species was found whereas esterase activity was only detected in *Rh. diobovatum*, *T. japonicum* and *Cryptococcus* species. The yeast *Y. lipolytica*, frequently isolated from fresh beef and fermented sausages, was reported to have a high technological potential due to its lipolytic and proteolytic activities (Gardini et al., 2001; Patrignani et al., 2007). Other yeast species such as *C. deformans*, *Cryp. kuetzingii*, *Cryp. curvatus*, *Rh. diobovatum*, *T. japonicum* and *T. montevideense* less often reported in the literature related to fermented sausages, also showed proteolytic and lipolytic abilities, suggesting that they also may influence flavor development.

In conclusion, to our knowledge this is the first report on the diversity of yeasts involved in the manufacturing process of llama meat fermented sausages. The most frequently isolated species were *D. hansenii* and *C. zeylanoides*. Moreover, the genotypic diversity found among strains within these dominant species is in agreement with the presence of several strains coexisting during the fermentation and ripening stages of llama sausage production. Among the isolated yeasts, strains of *D. hansenii* and *C. zeylanoides* as well as *Cryp. curvatus*, *Rh. diobovatum* and *Y. lipolytica* showed enzymatic activities technologically appropriate. Consequently, these indigenous yeasts may play an important role in the development of organoleptic characteristics of fermented and cured llama sausages; their selection as starter cultures may enhance llama sausage quality while preserving the territorial attributes of this product.

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