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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,

UK) in combination with the QuBit™ fluorometer. The bacterial V3-V4 16S rRNA region was amplified with the primer pairs 343F (5′-TACGGRAGGCAGCAG-3′) and 802R (5′-TACNVGGGTWTCTAATCC-3′) using Phusion Flash High-Fidelity Master Mix (Thermo Fisher Scientific, Inc. Waltham, MA, USA). A two-step nested-PCR was applied and conditions used for reaction mix and amplification experiments were those described by Vasileiadis et al. (2015). The PCR products of the second step for all samples were multiplexed in a single pool in equimolar amounts based on the QuBit quantification data. The PCR products pool was then purified using the solid phase reverse immobilization method of the Agencourt® AMPure® XP kit (Beckman Coulter, Italy, Milano) and sequenced at Fasteris SA (Geneva, Switzerland). The TruSeq ™rDNA sample preparation kit (Illumina Inc., San Diego, CA) was used for the amplicon library preparation, while the sequencing reaction was performed with a MiSeq Illumina instrument (Illumina Inc., San Diego, CA) with V3 chemistry, generating 300 bp paired-end reads.

2.6. Sequences data preparation and analyses

High-Throughput Sequencing data filtering, multiplexing and preparation for statistical analyses were carried out as previously described (Bassi et al., 2015; Połka et al., 2015; Vasileiadis et al., 2015). Briefly, paired-reads were assembled to reconstruct the full V3–V4 amplicons with the "PANDAseq" script (Masella et al., 2012) allowing a maximum of 2 mismatches and at least 30 bp of overlap between the read pairs. Samples demultiplexing was then carried out with the Fastx-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Mothur v.1.32.1 (Schloss et al., 2009) was applied to remove sequences with large homopolymers (≥10), sequences that lack alignment within the targeted V3–V4 regions, chimeric sequences (Edgar et al., 2011) and sequences that were not classified as bacterial after alignment against the Mothur version of the RDP (Ribosomal Database Project) training data set. Diversity indexes were calculated with Mothur using default parameters and average method in the clustering step. Consequently, the Observed (Sobs) and Chao's richness, and Shannon's diversity as well as evenness indexes were computed from OTUs clustered at 97% of similarity, which were obtained after processing a high quality and a normalized subset of 19,799. For taxonomy-based analyses, sequences were hierarchically classified in different taxa using the Greengenes database (McDonald et al., 2012). A manual amendment of the database was performed by adding all RDP deposited sequences of all species level sequences available for type strains belonging to the mostly represented genera in sausage samples (Połka et al., 2015). All statistical analyses and pairwise comparisons among groups were conducted on R v 3.1.

Raw sequences in read-pairs format were submitted to the National Centre for Biotechnology Information (NCBI) Sequence Read Archive.

2.7. Technological characterization of Lb. sakei isolates

2.7.1. Acidification and effect of temperature, NaCl and pH at 15 °C

Growth was evaluated measuring the OD at 15 °C in MRS broth by inoculation with a 1% active culture (final $OD_{560} = 0.2$) of each strain investigated. The acidifying capacity of strains was assayed during incubation at 15 °C by reading pH at 48 h. The ability to grow at pH 4.5 was determined in MRS adjusted with HCl (0.1 M) by using a pH-meter (Crison Instruments, Barcelona, Spain). The effect of NaCl on *Lb. sakei* growth was evaluated in MRS supplemented with 5.0, 7.5 and 10% NaCl and incubated at 15 °C.

2.7.2. Proteolytic activity

The extracellular protease/peptidase activity of *Lb. sakei* strains was determined using myofibrillar or sarcoplasmic pork proteins extracts prepared as reported by Fadda et al. (1999). Proteolytic activity was assayed on agar plates containing proteins extracts according to the protocol described by Mauriello et al. (2002). Briefly, after solidification of the medium, 70 μl of a 24-h culture broth of each strain were added into wells cut in the agar plates. After 48 h of incubation, the agar layers around the wells were removed and stained for 5 min in 0.05% (w/v) Brilliant Blue R (Sigma) in methanol:acetic acid:distilled water (50:10:40). After destaining (methanol:acetic acid:water; 25:5:70), the presence of a clear zone surrounding the inoculated wells indicated proteolytic activity and was expressed as positive (+) and the absence of halo as negative (−).

2.7.3. Lipolytic activity

Tributyrin agar (Oxoid) and PCA pH 5,5 supplemented with 4% of pork fat plates (De Pedro et al., 1997) were used to asses lipolytic activity of *Lb. sakei* strains as reported by Benito et al. (2007). The occurrence of clear zone surrounding *Lb. sakei* spots on the plates after 48 h of anaerobic incubation at 30 °C was considered as positive lipolytic activity.

2.7.4. Detection of the katA gene encoding catalase production

PCR amplification of a 410 bp from heme-dependent catalase gene (*katA*) was performed using DNA from *Lb. sakei* strains according to the primers and conditions described by Ammor et al. (2005).

2.7.5. PCR analysis for bacteriocin-encoding genes

All selected strains were tested by PCR to search for the presence of sakacin P (*sppA*), sakacin Q (*sppQ*) and curvacin A (*sapA*) encoding genes. Primers and PCR conditions used for each structural genes are those reported by Fontana et al. (2015). The amplified product was visualized in a 1.5% (w/v) agarose gel stained with SYBR Safe (Invitrogen, Italy) or GelRel (Biotium-Genbiotech, Argentina). All PCR runs included a blank control consisting of PCR-grade water and a non-template control (no DNA). All PCR products were purified and subjected to sequencing at CERELA Sequencing Service and BMR Genomics (Padova, Italy). The resulting sequences were analyzed with the NCBI Blast program (Altschul et al., 1997).

2.7.6. Biogenic amines production

Biogenic amines (BA) production was tested according to Bover-Cid and Holzapfel (1999). Briefly, the active culture of *Lb. sakei* strains were streaked on the decarboxylase medium plates containing the corresponding amino acid precursor at 1% final concentration (L-histidine monohydrochloride, tyrosine disodium salt, L-ornithine monohydrochloride and lysine monohydrochloride (Sigma-Aldrich, Steinheim, Germany). and incubated anaerobically at 37 °C for 4 days. Growth of decarboxylating strains was easily recognizable because of their yellow halo in the medium. Plates without amino acids were used as control.

2.7.7. Antibiotic resistance and MIC determination

Antibiotic resistance of strains and minimal inhibitory concentration (MIC) for ampicillin (Amp), chloramphenicol (Chl), clindamycin (Cli), erythromycin (Ery), gentamicin (Gen), tetracycline (Tet), Kanamycin (Kan) and Streptomycin (Str) were determined by using micro dilution technique according to EFSA Guidance (2012).

2.8. Statistical analyses of culture-based data

Viable bacterial counts were carried out in triplicate and microbiological data are presented as average values. A correlation analysis among the main microbial populations was performed and Pearson coefficient (PC) was calculated for each case with a significance of $p < 0.05$. Data processing was carried out by MINITAB (version 14) and Infostad (2015p Version) software.

3. Results and discussion

3.1. Bacterial communities present during llama sausages fermentation as determined by HTS

Illumina HTS technology was applied to achieve a detailed resolution of the total bacterial communities associated to both llama sausage productions at three different sampling time. The taxonomical assignment showed that the 99.4% of sequences were correctly classified at genus level and 94.4% at the species level. The OTU data were filtered with a cut-off of 99.9%. The coverage analysis and measurements of α-diversity indexes are reported per each sample in Table 1. There was satisfactory coverage of the diversity within both sausages productions; the highest diversity and richness being associated with llama sausages A production. In Fig. 1, samples clustered together by production technology show high differences at different taxonomical levels. When the relative abundance was calculated at phylum level (Fig. 1a), it was observed that Firmicutes largely predominated in P production; while Firmicutes and Proteobacteria were the main phyla detected in A production. The distribution of the main families at the three selected times along the sausages manufacturing processes (Fig. 1b) showed a prevalence of *Lactobacillaceae* and *Leuconostocaceae* during P production, whereas A production was characterized by additional *Pseudomonadaceae* and *Moraxellaceae* populations (taxa with lower participation were grouped as "other" sequences). Sequence assignment at genus level confirmed *Lactobacillus* and *Leuconostoc* as the most representative genera during P production, with a minor presence of *Staphylococcus* increasing in the T14 and T21 days samples (Fig. 2). A different microbiota picture was observed for A production where the presence of *Lactobacillus*, *Pseudomonas*, *Acinetobacter* and *Leuconostoc* genera were determined (Fig. 2). The high diversity observed at time 0 was maintained until the end of fermentation indicating that the selection of a dominant microbiota did not occur.

The analysis of the bacterial community at species level during both manufacturing processes showed that among technological bacteria, the most abundant species was *Lb. sakei* (Fig. 3). Concerning *Staphylococcus* population, *S. saprophyticus* became evident at the end of fermentation only for P production, being almost absent during A fermentation. The predominance and dynamics of this species was in accordance with those reported for pork Italian salami by Połka et al. (2015). Different *Leuconostoc* species were identified varying in their abundance during ripening; *Lc. inhae* and *Lc. mesenteroides* were present in both productions while *Lc. lactis* was only detected in P production. As shown in the heat-map (Fig. 3), other LAB such as *Weissella ghanensis*, *Weissella viridescens* and *Carnobacterium viridans* were mostly detected in A production whereas sequences belonging to *Enterococcus casseliflavus* were only identified from P production. Abundant groups of Gram-negative such as *Pseudomonas* spp., *Psychrobacter* spp., *Acinetobacter* spp. and enterobacteria were detected during A production; these spoilage microorganisms being frequently involved as undesirable bacteria in food processing environments. In particular, *Pseudomonas* could be part of the resident microbiota of food processing plants, this being facilitated by their well-known ability to adhere to food processing surfaces (Stellato et al., 2015). In this study, the low ripening temperature during A production and the psychrotrophic nature of *Pseudomonas* could help it to withstand the competing microbial populations during sausages ripening.

In the present study, HTS was used to analyze diversity and evolution of bacterial populations at different time during fermentation, this sequencing approach being useful to identify the total bacterial ecology in two different llama sausage productions. The dynamics of identified species throughout both manufacturing process, clearly reflected as the environmental and production conditions highly influence bacterial composition of the meat product. An important piece of evidence was the presence of *Lb. sakei* during both productions confirming its technological role during llama meat fermentation process. These results extend the knowledge about LAB participation in sausages fermentation being the first report about bacterial ecology of llama meat sausages. HTS approach has become an essential tool for in-depth analysis of the microbial diversity in food ecosystems (Ercolini, 2013; Petrosino et al., 2009). Recently, Połka et al. (2015) applied HTS to analyze the bacterial diversity in typical Italian salami demonstrating that this approach applied to food ecology allows gaining a complete picture of the bacterial species involved in the fermentation.

3.2. Microbiological analyses

Counts of total mesophilic (TM) bacteria, lactobacilli, enterococci and GCC+ were determined during the fermentation/ripening process of P and A llama sausages by applying scatterplot matrix (Fig. 4a, b respectively). Simple linear correlation (Pearson correlation, PC) was used as a measure of the associations among CFU changes in the different microbial populations. During P process, a high TM/lactobacilli correlation (PC = 0.96) was found during whole fermentation process; both populations reaching 7.4 and 6.6 log CFU/g, respectively at 28 days (Fig. 4a). Therefore, lactobacilli represented the major microbial group in the TM population during P fermentation. Although GCC+ group did not largely contributed to TM population, a positive TM/GCC+ correlation (PC = 0.79) was established after 4 days, with final counts of 5.5 log CFU/g. Enterococci, showed a decrease of their counts at the end of ripening, evidencing weak correlations with TM (PC = 0.30) and lactobacilli (PC = 0.44), while a higher enterococci/GCC+ correlation (PC = 0.60) was found. On the other hand, during A manufacturing, TM/lactobacilli correlation (PC = 0.66) was lower than in P production; with initial lactobacilli count of 6.6 log CFU/g decreasing to 5.7 log CFU/g at 20 days (Fig. 4b). Although positive correlations for GCC+/TM (PC = 0.84) and GCC+/lactobacilli (PC = 0.41) were found during A production, a dramatic reduction of GCC+ numbers after day 4 occurred. Negative correlations for enterococci with TM, GCC+ and lactobacilli (PC = −0.23, −0.21 and −0.31, respectively) were observed (Fig. 4b). When molds and yeasts were analyzed, a negative correlation with TM and other bacterial groups was determined for the P production (Fig. 4a); conversely, a significant positive correlation between molds/yeasts and TM (PC = 0.74) was obtained for A manufacturing (Fig. 4a, b).

Changes in pH observed during P production showed a reduction from 5.6 to 4.9 at 12 days of fermentation, however, an increase to a final value of 5.7 was then detected. On the other hand, a continuous reduction of pH from 6.0 to 5.1 at 20 days was registered for A production whereas similar a_w reductions (0.97 to 0.86) occurred for both sausage

Table 1
Number of sequences analyzed, estimated sample coverage for 16S rRNA amplicon, diversity richness (Chao 1) and diversity index (Shannon) for llama fermented sausages P and A productions.

Sample	nseqs	Coverage	Chao's richness index	Sobs	Shannon diversity index	Shannon evenness index
P day 0	19,799	99.64%	265.333	147	1.390	0.273
P day 14	19,799	99.60%	294.050	140	0.862	0.169
P day 21	19,799	99.67%	221.800	121	0.994	0.202
A day 0	19,799	99.66%	278.000	135	1.837	0.370
A day 10	19,799	99.65%	277.333	147	2.156	0.428
A day 20	19,799	99.70%	222.833	131	2.085	0.424

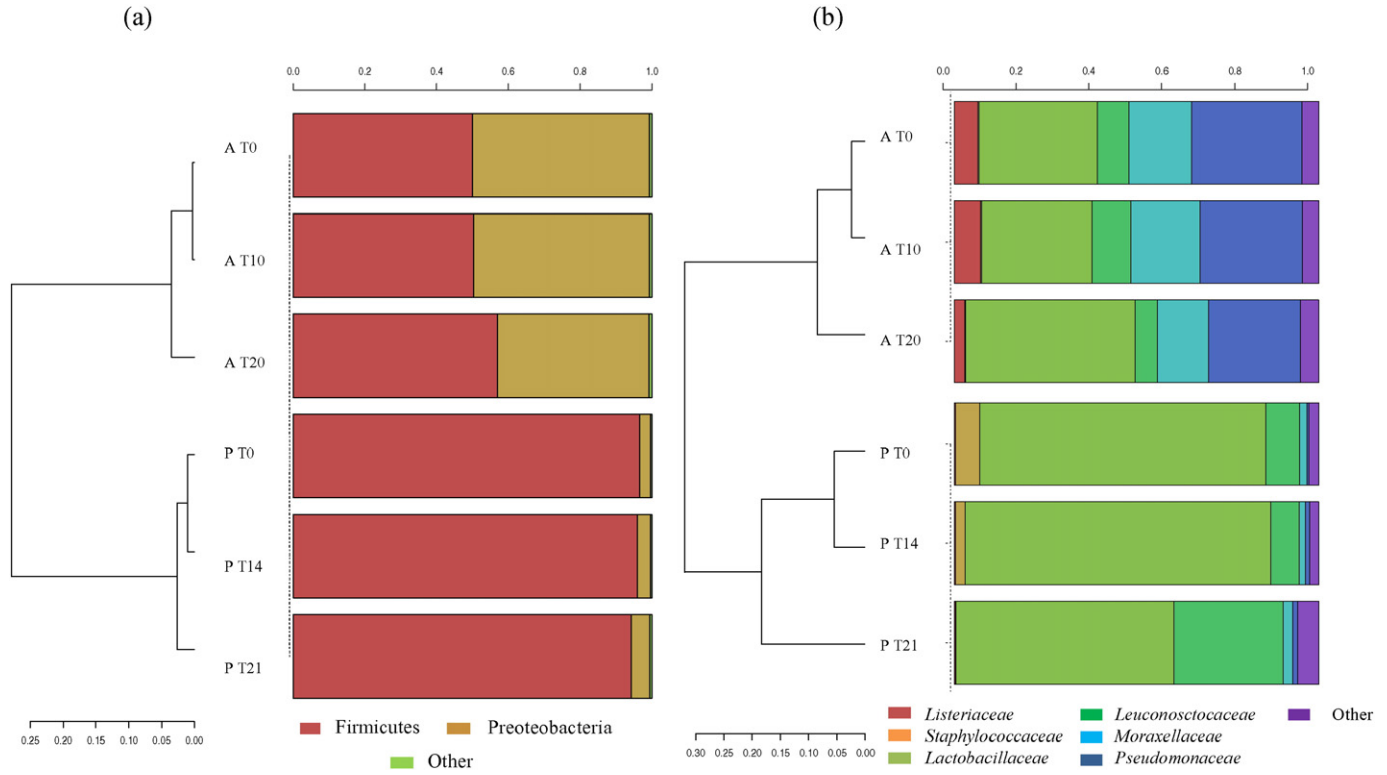


Fig. 1. Hierarchical clustering of classified sequences using the average linkage algorithm according to the phylum (a) and family (b) classifications for taxa participating with ≥5% in at least one sample. Taxa with lower participations were added to the "other" sequence group. Samples from artisanal "A" and pilot production "P" at three different time during ripening are shown.

productions (data not shown). Similar pH values were recorded for Iberian dry-cured sausages, which are typical of low-acidic fermented products (Aymerich et al., 2003; Benito et al., 2007); reductions of a_w during both llama sausage productions were in agreement to those found for Italian low-acid sausages (Spaziani et al., 2009), but higher to analogous Iberian products (Benito et al., 2007). Concerning pathogens, high counts of coliforms were found initially, decreasing their numbers to less than 10^2 CFU/g at the end of processing, whereas *L. monocytogenes* and *Staphylococcus aureus* were absent in both productions (data not shown).

In coincidence with HTS results described above, microbiological analysis confirmed the relevant role played by lactobacilli in the

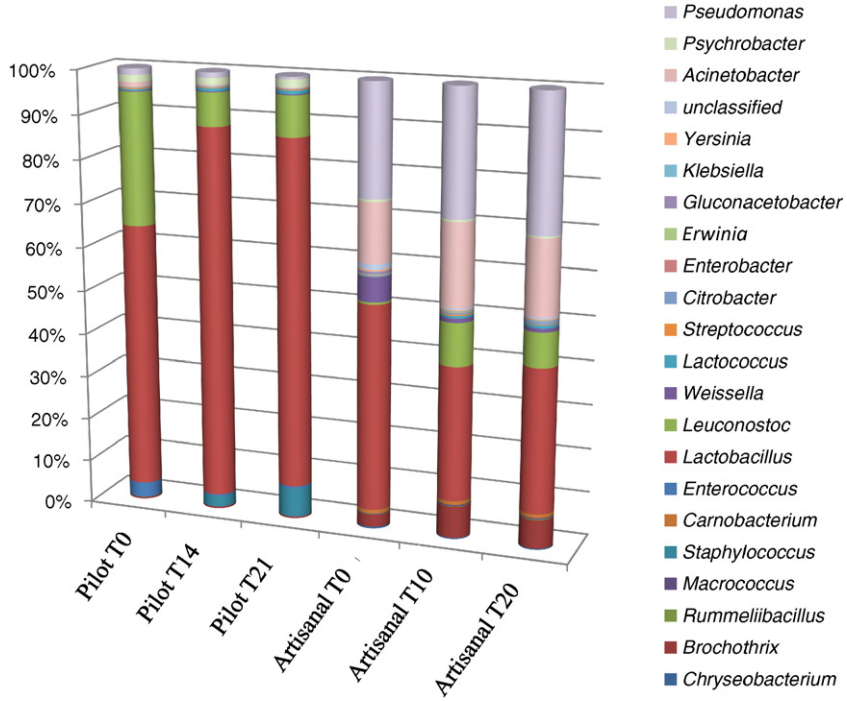


Fig. 2. Identification of bacterial populations in sausage productions from (pilot) and (artisanal) derived from NGS analysis. Relative abundance in genera at three different times is shown.

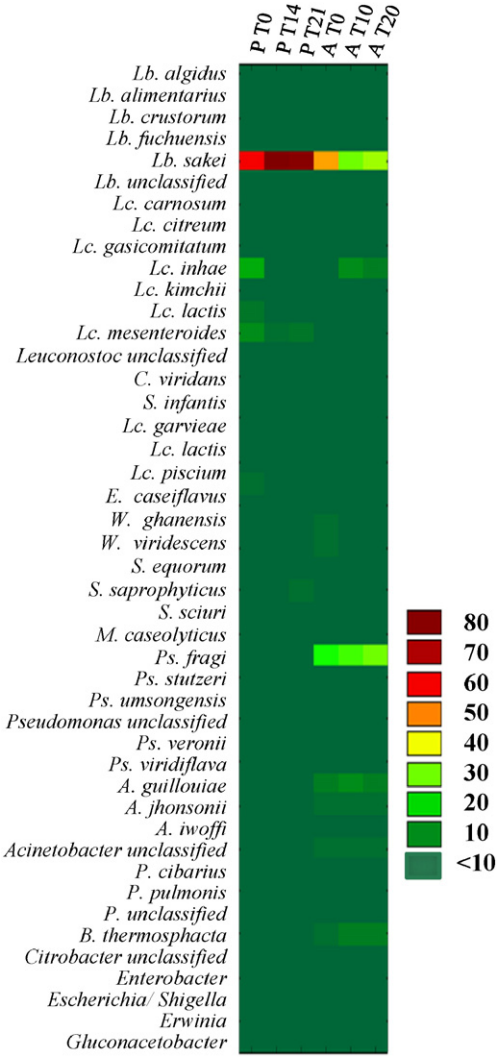


Fig. 3. Heat map showing species abundance and distribution during the manufacturing processes of pilot (P) and artisanal (A) sausages production. Species accounting for >0.1% are represented.

ripening process as was previously reported for pork sausages (Albano et al., 2009; Fontana et al., 2005; Palavecino Prpich et al., 2015). Concerning GCC + group, growth dynamics differences obtained by culture-based analysis in both productions were reflected by HTS analysis. The decrease in GCC + numbers after 4 days during A fermentation was in coincidence with the absence of sequences detected by HTS for this group; on the contrary the increase of GCC + counts after day 7 for P production allowed detecting sequences for this population by HTS.

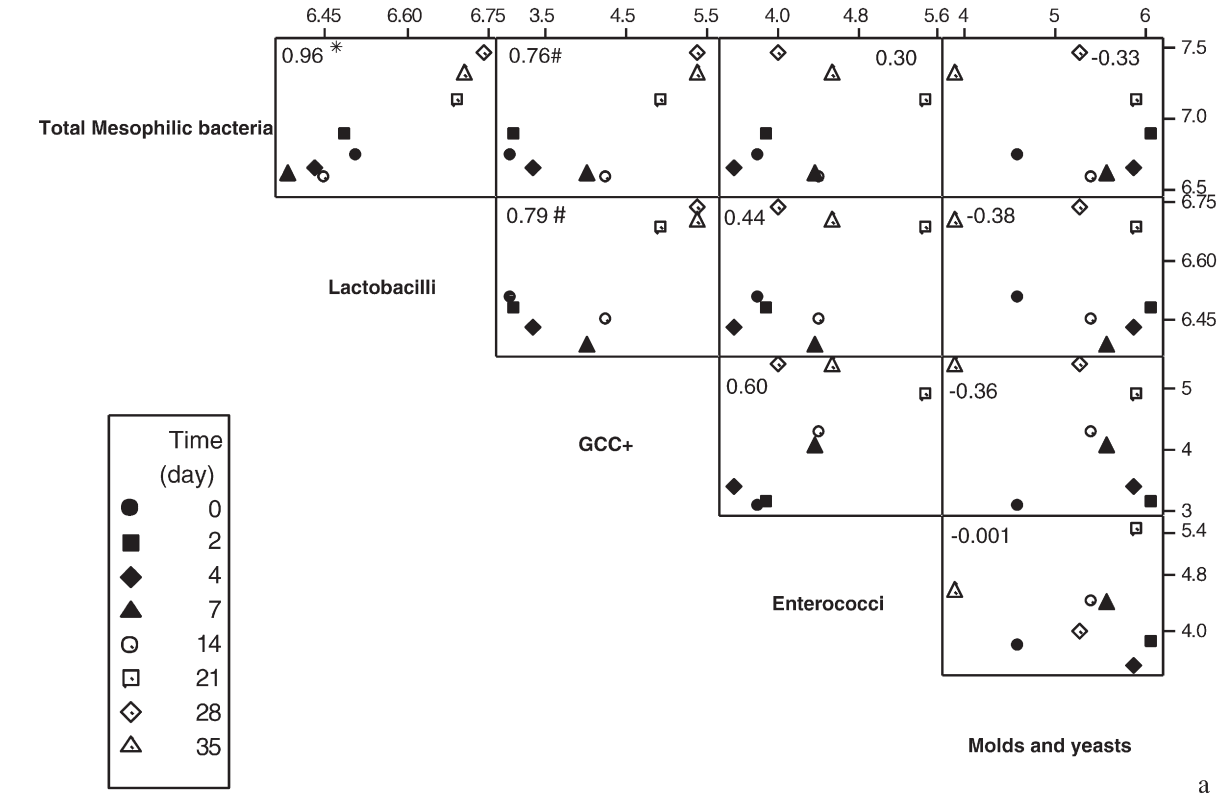
3.3. Lactobacilli identification and dynamics during llama sausage productions

A total of 230 presumptive LAB (160 and 70 from P and A productions, respectively) were isolated at different times during ripening from MRS and Rogosa plates. To investigate the bacterial diversity, each isolate was primary differentiated by means of RAPD. RAPD fingerprints for P production showed a high variability at the early stages of fermentation (0–14 days); the effect of growth dynamics and ripening conditions resulted in the predominance of a single profile at the end of the fermentation process (Fig. S1a). The RAPD picture for A production reveled that isolates were genotypically more heterogeneous during the whole fermentation/ripening process, with no strain being particularly selected (Fig. S1b). Taxonomical identification by means of sp-sp PCR revealed *Lb. sakei* as the sole species recovered from P production, whereas a few *Lb. plantarum* strains were also identified from A production (Fig. S1a, b). As *Lb. sakei* largely dominated both fermentation processes, the genotypic relatedness among these strains (24 and 14 for P and A productions, respectively) was investigated by RAPD cluster analysis. The dendrogram shown in Fig. 5 revealed the presence of clusters I and II at a similarity level of 75%; cluster I (subdivided into 3 minor clusters) involved *Lb. sakei* strains from both productions whereas cluster II grouped only strains from P production. At similarity of 70%, cluster III contained strains from P production and additionally, *Lb. sakei* strains UC10278, UC10279, UC10274 and UC10284 are grouped more distant from the others strains. *Lb. sakei* is recognized to play a major role during traditional and industrial dry sausages fermentation, representing the major population (Ammor et al., 2005; Bonomo et al., 2008; Cocolin et al., 2009; Fontana et al., 2005; Palavecino Prpich et al., 2015; Urso et al., 2006). In this study, cluster analysis of the obtained patterns evidenced the presence of different biotypes for identified *Lb. sakei* strains. Intra-species identification and characterization constitute useful tools to distinguish groups of strains or single strains with peculiar technological properties for innovative biotechnological processes. Indeed, cultivation and isolation of microorganisms is still deemed as strictly necessary in certain domains of investigation, particularly in food microbiology for the development of starter cultures as recently stated by Cocolin and Ercolini (2015).

3.4. Technological and safety characterization of isolates

The main purpose of LAB used in meat fermentation as starter cultures is to enhance the product sensory and safety characteristics as well as to extend its shelf life. In order to select strains for the production of traditional llama sausages, technological features (salt and pH tolerance, acidification and growth at 15 °C, proteolytic and lipolytic activity) of 40 *Lb. sakei* strains were evaluated. A summary of the main results obtained from technological and safety characterization of *Lb. sakei* strains and the relationships among phylogenetic clusters previously described is shown in Fig. 5. *Lb. sakei* acidifying ability at 15 °C (fermentation average temperature) was variable; after 48 h, 37 out of 40 strains decreased their pH to 4.05–4.88, while the remaining 3 strains from A production exhibited pH values above 5.00. The highest pH reductions were observed for P production *Lb. sakei* strains while most of the strains were able to grow at pH 4.5. Concerning to NaCl tolerance at 15 °C, results showed 42.5% of *Lb. sakei* strains were able to grow in the presence of 5% NaCl, most of them being from A production; higher salt concentration resulted in a weak *Lb. sakei* strains growth. Similar results were reported for strains isolated from French and Greek traditional dry fermented sausages (Ammor et al., 2005; Papamanoli et al., 2003). Hydrolytic activity on myofibrillar and sarcoplasmic proteins was evidenced in 72.5% and 80% of *Lb. sakei* strains respectively, whereas 57.5% of them degraded both meat protein fractions. Although LAB have only weak proteolytic effect on meat proteins, *Lb. sakei* has been reported to exert peptidogenic and aminopeptidase activity during the ripening of fermented sausages with flavor impact (Fadda et al., 1999, 2010). No lipolytic activity was found among *Lb. sakei* strains analyzed (data not shown). *Lactobacillus* species have been reported to be weakly lipolytic; bacterial lipases seem to be ineffective under conditions prevailing during sausage fermentation (Villani et al., 2007). On the other hand, when the presence of *kat*A gene in *Lb. sakei* strains was investigated, 85% of strains exhibited a PCR product of the expected size suggesting the presence of this gene (Fig. 5), in coincidence with those reported by Ammor et al. (2005).

PCR evaluation for the bacteriocin-encoding genes showed 14 out of 40 examined strains (35%) mostly from A production, harboring structural bacteriocins genes. Sakacin P (*spa*) gene was detected for 12 strains, sakacin Q (*sppQ*) and curvacin (*sapA*) were harbored by *Lb. sakei* UC10338 and UC10284 respectively, whereas *Lb. sakei* UC10356 carried the genes for the production of sakacin P and sakacin Q. The



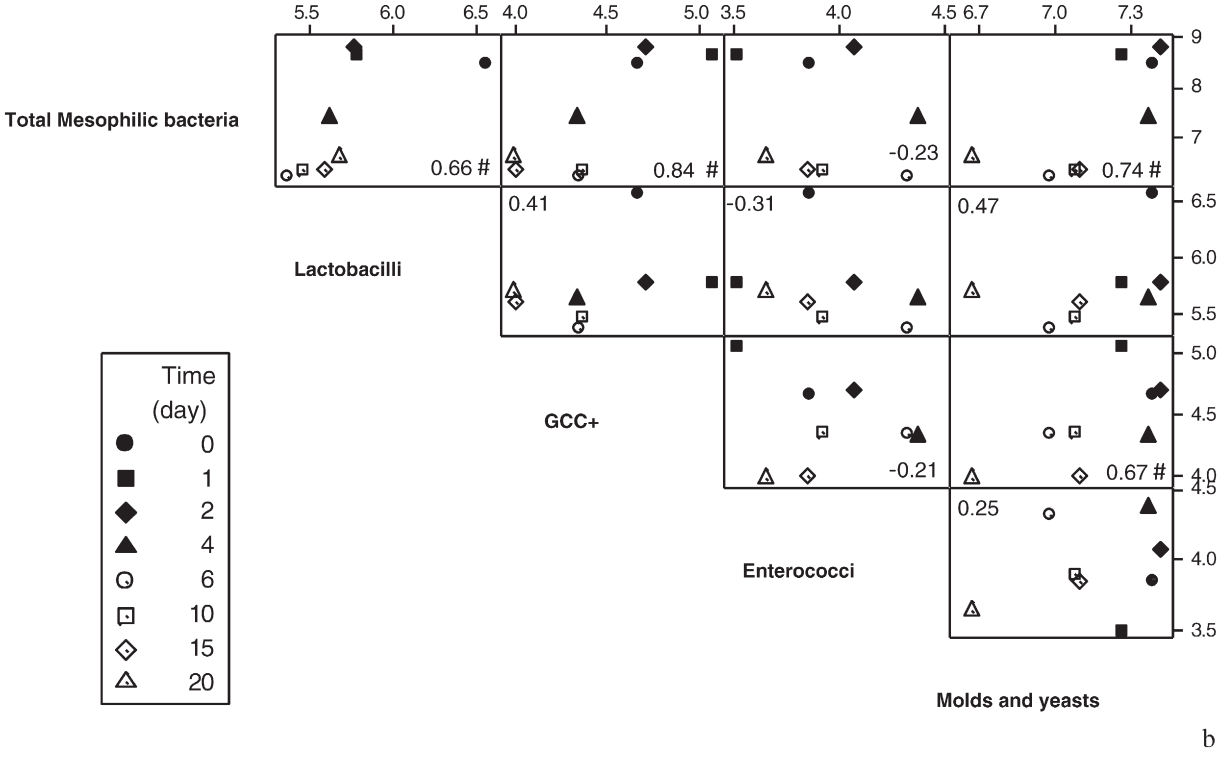
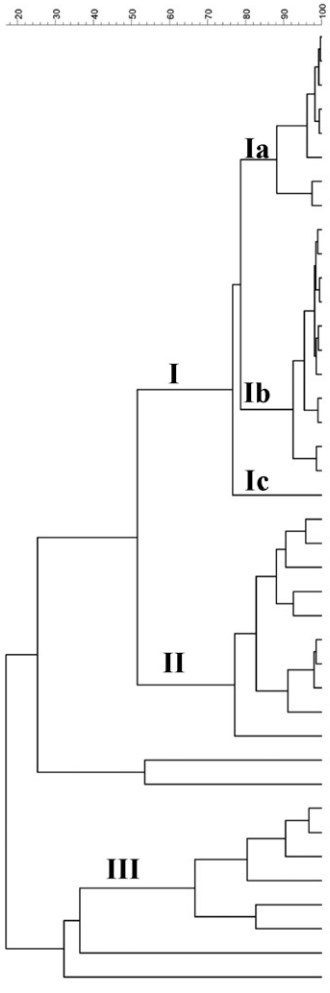


Fig. 4. Bacterial growth kinetic during llama meat fermentation, a) pilot scale and b) artisanal productions. The relationship between the log CFU/g of the most relevant microbial groups with technologic implications is shown. Pearson coefficient and the significance of correlation are indicated by * ($p \leq 0.001$) and # ($p \leq 0.05$).

high occurrence of curvacin A, sakacin Q and P bacteriocin genes in *Lb. sakei* strains with great effectiveness against *Listeria* were recently reported (Fontana et al., 2015).

Besides the traditional properties for strains competitiveness under sausage fermentation conditions, for novel starter cultures, the risks posed by biogenic amines formation, and bacterial resistance to antibiotics (Ammor and Mayo, 2007) should be considered. The aminogenic potential of the investigated *Lb. sakei* strains was negative. In agreement, Landeta et al. (2013) reported low number of aminogenic *Lb. sakei* strains from pork sausages. Regarding to antibiotic resistance, all



	Sausage production		Acid production (pH at 48h)	Proteolytic activity		*Growth at pH 4.5	*NaCl tolerance at 15 °C			**katA gene	**Bacteriocin encoding gene	***Antibiotic resisitance
	Pilot plant	Artisanal		Myofib.	Sarcopl.		5%	7,5%	10%			
UC10265			4.10	+	+	+	+	w	w	+	-	Gen/Str
UC10264			4.80	+	+	+	+	w	w	+	-	-
UC10272			4.48	-	-	+	w	w	w	+	-	-
UC10281			4.05	+	-	+	w	w	w	+	-	Kan/Str
UC10273			4.35	+	-	+	w	w	w	+	-	Str
UC10338			4.56	+	+	+	+	w	w	-	*sppQ*	-
UC10348			4.88	+	+	+	+	w	w	+	*sppA*	-
UC10349			4.78	+	+	+	+	w	w	+	-	-
UC10353			4.13	+	+	+	+	w	w	+	*sppA*	-
UC10346			5.89	+	+	+	+	w	w	+	*sppA*	Amp/Kan
UC10355			4.66	+	+	+	+	w	w	+	*sppA*	-
UC10341			4.34	+	+	+	+	w	w	+	*sppA*	-
UC10354			4.51	+	+	+	+	w	w	+	-	-
UC10356			5.01	+	+	-	-	-	-	+	*sppA*/*sppQ*	-
UC10339			4.78	+	+	+	+	w	w	+	*sppA*	Kan
UC10343			5.05	+	+	+	+	w	w	+	*sppA*	-
UC10342			4.20	+	+	+	+	w	w	+	*sppA*	-
UC10347			4.40	+	+	+	+	w	w	+	-	-
UC10350			4.88	+	+	+	+	w	w	+	*sppA*	-
UC10325			4.77	-	+	+	w	w	w	+	-	Str
UC10323			4.20	+	-	+	+	+	w	+	-	Gen/Str
UC10280			4.23	+	-	+	w	w	w	+	-	Str
UC10276			4.10	-	+	+	+	w	w	+	-	Kan/Str
UC10266			4.54	-	+	+	w	w	w	-	-	Str
UC10267			4.35	+	+	+	w	w	w	+	-	Kan/Str/Chl
UC10277			4.10	-	+	+	w	w	w	+	-	Str
UC10269			4.54	+	+	+	w	w	w	+	-	Str
UC10261			4.55	+	+	-	w	w	w	+	-	-
UC10285			4.39	-	+	+	w	w	w	+	*sppA*	Gen/Str
UC10324			4.35	+	-	+	w	w	w	-	-	Str
UC10279			4.82	+	+	+	+	+	+	+	-	Str
UC10278			4.76	+	+	+	w	w	w	+	*sppA*	Str
UC10262			4.39	-	-	+	w	w	w	+	-	Gen/Str
UC10268			4.23	-	+	+	w	w	w	+	-	-
UC10326			4.45	+	+	+	w	w	w	+	-	Str
UC10271			4.55	-	+	+	w	w	w	+	-	-
UC10270			4.47	-	+	+	w	w	-	-	-	Str
UC10263			4.60	+	+	+	w	w	w	-	-	Str
UC10274			4.46	+	-	+	-	-	-	-	-	Chl
UC10284			4.56	-	+	+	w	w	w	+	*sapA*	Gen/Str

Fig. 5. Cluster analysis of the profiles obtained by RAPD-PCR from the *Lb. sakei* strains isolated from the naturally fermented llama sausages isolated from pilot plant (P) and artisanal (A) productions (clusters labelled I to IV). Technological, functional and safety properties of isolates are reported in the table: *(−) $OD_{600} \leq 0.5$; (+) $0.5 \leq OD_{600} \geq 1.0$; w (weak): $0.5 \leq OD_{600} \geq 1.0$; **PCR-gene amplification: *sppA* (sakacin A), *sppQ* (sakacin Q), *sapA* (curvacin A). ***Antibiotic resistance: ampicillin (Amp), chloramphenicol (Chl), clindamycin (Cli), erythromycin (Ery), gentamicin (Gen) tetracycline (Tet), Kanamycin (Kan), Streptomycin (Str).

strains were sensitive to erythromycin, tetracycline and clindamycin, whereas *Lb. sakei* UC10274 and UC10339 were resistant to chloramphenicol and Kanamycin, respectively. Resistance to Streptomycin (30%), double resistance to Gen/Str (12.5%), Kan/Str (5%), Amp/Kan (2.5%) and a mutiresistance against Kan/Str/Chl (2.5%) were found, whereas 17 *Lb. sakei* strains (42.5%) were sensitive to all assayed antibiotics. Similar high susceptibility to Ery, Tet, Cli, Gen and Amp was described for strains isolated from Spanish and Italian fermented sausages (Aymerich et al., 2006; Landeta et al., 2013; Zonenschain et al., 2009). Notably, *Lb. sakei* isolates from P production presented a high prevalence of antibiotic resistance, when compared to A production, while *Lb. sakei* strains isolated from A production, even when they showed lower acidification rates, exhibited high presence of genes coding for bacteriocins and a lower prevalence of antibiotic resistance.

4. Conclusions

In this work, the use of more than one methodology (HTS and culture-based approach) to investigate the microbial biodiversity of llama fermented sausages, provided a better global overview of its microbial composition. HTS offered the advantage of describing in detail subpopulations at the species level, which are hardly highlighted through culture-dependent approaches due to inherent limitations. Moreover, isolation and monitoring microbes beyond the species-level identification is crucial to assess the performance and fitness of strains to be used as starter cultures. The selection of autochthonous *Lb. sakei* strains from llama sausages, based on technological and safety criteria would allow to obtain a functional starter culture better adapted to this traditional product and could help to preserve its features of uniqueness. The characterization of this specific ecological niche will provide protection to the increased consumption of llama sausage and their contribution to local economy.

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