Pyrosequencing vs. culture-dependent approaches to analyze lactic acid bacteria associated to *chicha*, a traditional maize-based fermented beverage from Northwestern Argentina

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ABSTRACT

The diversity of lactic acid bacteria (LAB) associated with *chicha*, a traditional maize-based fermented alcoholic beverage from Northwestern Argentina, was analyzed using culture-dependent and culture-independent approaches. Samples corresponding to 10 production steps were obtained from two local producers at Maimará (*chicha* M) and Tumbaya (*chicha* T). Whereas by culture-dependent approach a few number of species (*Lactobacillus plantarum* and *Weissella viridescens* in *chicha* M, and *Enterococcus faecium* and *Leuconostoc mesenteroides* in *chicha* T) were identified, a higher quantitative distribution of taxa was found in both beverages by pyrosequencing. The relative abundance of OTUs was higher in *chicha* M than in *chicha* T; six LAB genera were common for *chicha* M and T: *Enterococcus*, *Lactococcus*, *Streptococcus*, *Weissella*, *Leuconostoc* and *Lactobacillus* while *Pediococcus* only was detected in *chicha* M. Among the 46 identified LAB species, those of *Lactobacillus* were dominant in both *chicha* samples, exhibiting the highest diversity, whereas *Enterococcus* and *Leuconostoc* were recorded as the second dominant genera in *chicha* T and M, respectively. Identification at species level showed the predominance of *Lb. plantarum*, *Lactobacillus rossiae*, *Leuconostoc lactis* and *W. viridescens* in *chicha* M while *Enterococcus hirae*, *E. faecium*, *Lc. mesenteroides* and *Weissella confusa* predominated in *chicha* T samples. In parallel, when presumptive LAB isolates (*chicha* M: 146; *chicha* T: 246) recovered from the same samples were identified by ISR-PCR and RAPD-PCR profiles, species-specific PCR and 16S rRNA gene sequencing, most of them were assigned to the *Leuconostoc* genus (*Lc. mesenteroides* and *Lc. lactis*) in *chicha* M, *Lactobacillus*, *Weissella* and *Enterococcus* being also present. In contrast, *chicha* T exhibited the presence of *Enterococcus* and *Leuconostoc*, *E. faecium* being the most representative species. Massive sequencing approach was applied for the first time to study the diversity and evolution of microbial communities during *chicha* manufacture. Although differences in the LAB species profile between the two geographically different *chicha* productions were observed by culturing, a larger number for predominant LAB species as well as other minorities were revealed by pyrosequencing. The fine molecular inventory achieved by pyrosequencing provided more precise information on LAB population composition than culture-dependent analysis processes.

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1. Introduction

A great variety of traditional fermented foods and beverages are produced in African, Asian, American and East European countries, many of them from cereals, leguminous plants, root tubers and fruits (Abriouel et al., 2006; Botes et al., 2007; Chen et al., 2006; De Vuyst et al., 2014; Escalante et al., 2008; Lacerda Ramos et al., 2010; Schoustra et al., 2013; Yousif et al., 2010) involving mixed cultures of bacteria, yeasts and/or fungi (De Vuyst et al., 2014). Cereal fermented products, in particular those derived from maize, are very important in Latin America and have been consumed as main staple food for centuries. Some of them are, even today, consumed as stimulants or are used in traditional medicine and in religious ceremonies. Among them, *chicha* is the most important traditional fermented beverage, which is produced since pre-Hispanic times in north-west regions of Argentina, and Andean regions of Bolivia, Colombia, Ecuador and Peru. *Chicha* manufacture is a household and communal activity and is mainly consumed by the native population during religious and agricultural festivities as well as during family and social events (Delibes Mateos and Barragan Villena,

2008; Lorence-Quiñones et al., 1999). *Chicha* is produced following handmade ancestral procedures and is, to some extent, related to the brewery process, so that it is also called maize beer; its production has not changed very much over the centuries (Steinkraus, 2002). *Chicha* is produced using a great variety of procedures depending on the maize varieties employed, utensils and local traditions. As starch hydrolysis is an essential step, methods used for maize flour treatment vary rather widely. Traditionally, the production of *chicha* involved saliva as inoculum that served as amylase source to convert starch into fermentable sugars. Nowadays, alternatives for amylase production are malting (germination) of maize kernels (*chicha de jora*) or adding a pre-fermentation step during manufacturing process. The resulting product is a clear, yellowish, effervescent and alcoholic (2–12% v/v) beverage produced using different local maize varieties (Lorence-Quiñones et al., 1999).

Previous studies on the microorganisms involved in the fermentation of *chicha de jora* revealed lactic acid bacteria (LAB) and yeasts as the dominant native populations. When characterized by physiological and biochemical techniques, *Lactobacillus* was predominantly identified (Quillama and Liendo, 1995). In addition, bacteria of the genus *Leuconostoc* were also reported as primary fermenting organisms (Steinkraus, 2002). In the last decade, the advent of culture-independent approaches have enlarged our knowledge on the microbiota composition of fermented food ecosystems enabling the detection of species that either are minority or require special culture conditions.

The aim of this study was to evaluate LAB populations present in two productions of *chicha*, a traditional alcoholic beverage prepared by local producers from Northwestern Argentina, by combining both culture-dependent and HTS approaches. Pyrosequencing of the V3–V5 16S rRNA gene and culture-dependent methods for LAB recovery were used to obtain information about the bacterial community, including LAB. Studied samples (*chicha* M and *chicha* T) were obtained from two local producers, each of them exhibiting particular manufacturing procedures. Physicochemical and microbiological parameters were determined for comparison purposes.

2. Materials and methods

2.1. Analysis and bacterial isolation

2.1.1. Chicha manufacturing process and sampling

Traditional *chicha* beverages were prepared by two different local producers from Northwestern Argentina (Quebrada de Humahuaca, Jujuy). Both traditional products were produced in the towns of Maimará and Tumbaya at 2400 and 2300 m above sea level, respectively, following the schematic flow-chart showed in Fig. 1. The process was a two-step fermentation using maize flour as substrate. For *chicha* M (Maimará production) "criollo" (ancient regional variety) maize flour was soaked with warm water (about 40 °C, approx. 1:1, w/v) and thoroughly mixed until a semi-solid dough was obtained. After cooling the mixture, flat buns were hand shaped and cooked on a tray in a rudimentary clay oven for 1.5 h. For Tumbaya production, maize flour was toasted in a clay oven before being added with water. Toasting or baking allows Maillard reaction for the typical *chicha* brownish color and flavor. Baked buns and toasted flour were hand mixed with cane sugar and warm water (additional fresh "criollo" maize flour was also added for *chicha* M) and fermented at ambient temperature (18–20 °C) for 7–8 days. In both productions, commercial maize flour and warm water were added to the pre-fermented mass (*mucus*) and mixed thoroughly and the obtained slurry was allowed to cool and decant for 2–3 h. Three layers were then separated: a top liquid layer (supernatant), a middle jelly-like layer and a bottom residual layer (discarded). In Maimará production (*chicha* M), the middle layer was pressed, cloth filtered and simmered to caramelize (*arrope*). Then, it was mixed with the separated supernatant, added with more water and allowed to ferment for 2–

4 days at 20 °C. When bubbling ceased, it was transferred to narrow-mouth pots and was ready for consumption. On the other hand, for Tumbaya production (*chicha* T), fermentation for 72 h at 20 °C occurred in the separated top liquid layer, which was then mixed with the concentrated *arrope* and added with more water and the beverage was ready to be consumed. Ten samples from each *chicha* production (M and T) were taken as described in Table 1.

2.1.2. Physicochemical parameters

The pH was determined using a digital pH meter (PT-10 Sartorius) while titratable acidity, expressed as lactic acid %, was performed by titrating 10 g of each sample with 0.1 N NaOH using phenolphthalein as indicator. Glucose content was determined using a Glicemia enzymatic kit (Wiener Laboratories, Argentina) and a test kit maltose/sucrose/D-glucose (R-Biopharm, Darmstadt, Germany) was used for maltose and sucrose determinations. Ethanol content was assayed using the R-Biopharm AG kit, whereas starch content was determined by the enzymatic/colorimetric method as described by Tovar et al. (1990) which includes the incubation with Termamyl for 20 min at boiling temperature, digestion with amyloglucosidase at 60 °C (30 min), and free glucose measurement using the combined glucose oxidase/peroxidase colorimetric assay.

2.1.3. Microbiological analysis and lactic acid bacteria isolation

Samples (5 g) were aseptically homogenized in 45 ml of buffered peptone water in a sterile plastic bag with lateral filter (BagPage S 400, BagSystem, Interscience, St-Nom-la-Breteche, France) using a Pulsifier (Microgen Bioproducts, Surrey, UK) for 15 s. One milliliter of the resulting mixture was taken from the filter side and tenfold serially diluted in sterile saline solution (NaCl 0.9 w/v). Microbial suspensions were plated in triplicate and incubated as follows: total mesophilic counts on Plate Count Agar (PCA, Conda, Madrid, Spain) incubated aerobically at 30 °C for 72 h; mesophilic LAB on MRS agar containing glucose (MRS), maltose (MRS-M) or starch (MRS-S) at 0.5% (w/v) and Yeast Glucose Lactose Peptone (YGLP), incubated anaerobically at 30 °C for up to 7 days. MRS and YGLP were prepared as indicated in http://www.cect.org. Counts of total yeasts and molds were performed on yeast and mold agar (Britania, Buenos Aires, Argentina) incubated at 30 °C for 72 h. Total coliforms were counted on McConkey agar (Britania, Buenos Aires, Argentina) incubated at 37 °C for 48 h. Media for LAB isolation were supplemented with cyclohexamide (Sigma-Aldrich, Madrid, Spain) at 100 μg/ml, in order to prevent mold and yeast growth. Counts were performed in triplicate. Correlation coefficient of total counts (in PCA) and LAB counts (in MRS and YGLP) was calculated using the Correlation Coefficient Tool of Excel software. For each sample, up to six colonies per LAB medium representing different morphologies were randomly picked from plates and subcultured on the corresponding medium. Gram-positive (Gregersen, 1978) and catalase negative (determined by transferring fresh colonies from a Petri dish to a glass slide and adding H_2O_2 3%, v/v) bacteria were considered as presumptive LAB and were purified by successive subculturing. Selected strains were stored for a long term at −20 °C in a 10% (w/v) dilution of the corresponding broth medium supplemented with 20% (w/v) glycerol.

2.2. Culture-dependent analysis of LAB populations

2.2.1. Bacterial strains, growth conditions and DNA isolation

Reference cultures used in this work were supplied by the Spanish Type Culture Collection (CECT): *Enterococcus faecium* CECT 410[T], *Enterococcus gallinarum* CECT 970[T], *Enterococcus casseliflavus* CECT 969[T], *Enterococcus faecalis* CECT 481[T], *Lactobacillus brevis* CECT 4121[T], *Lactobacillus pentosus* CECT 4023[T], *Lactobacillus plantarum* CECT 748[T], CECT 6000, CECT 4674, CECT 4219, *Leuconostoc mesenteroides* subsp. *mesenteroides* CECT 219[T], *Lc. mesenteroides* subsp. *dextranicum* CECT 912[T], *Leuconostoc lactis* CECT 4173[T] and *Weissella viridescens* CECT 283[T]. They were routinely grown on MRS (Oxoid) at 28 °C and stored in
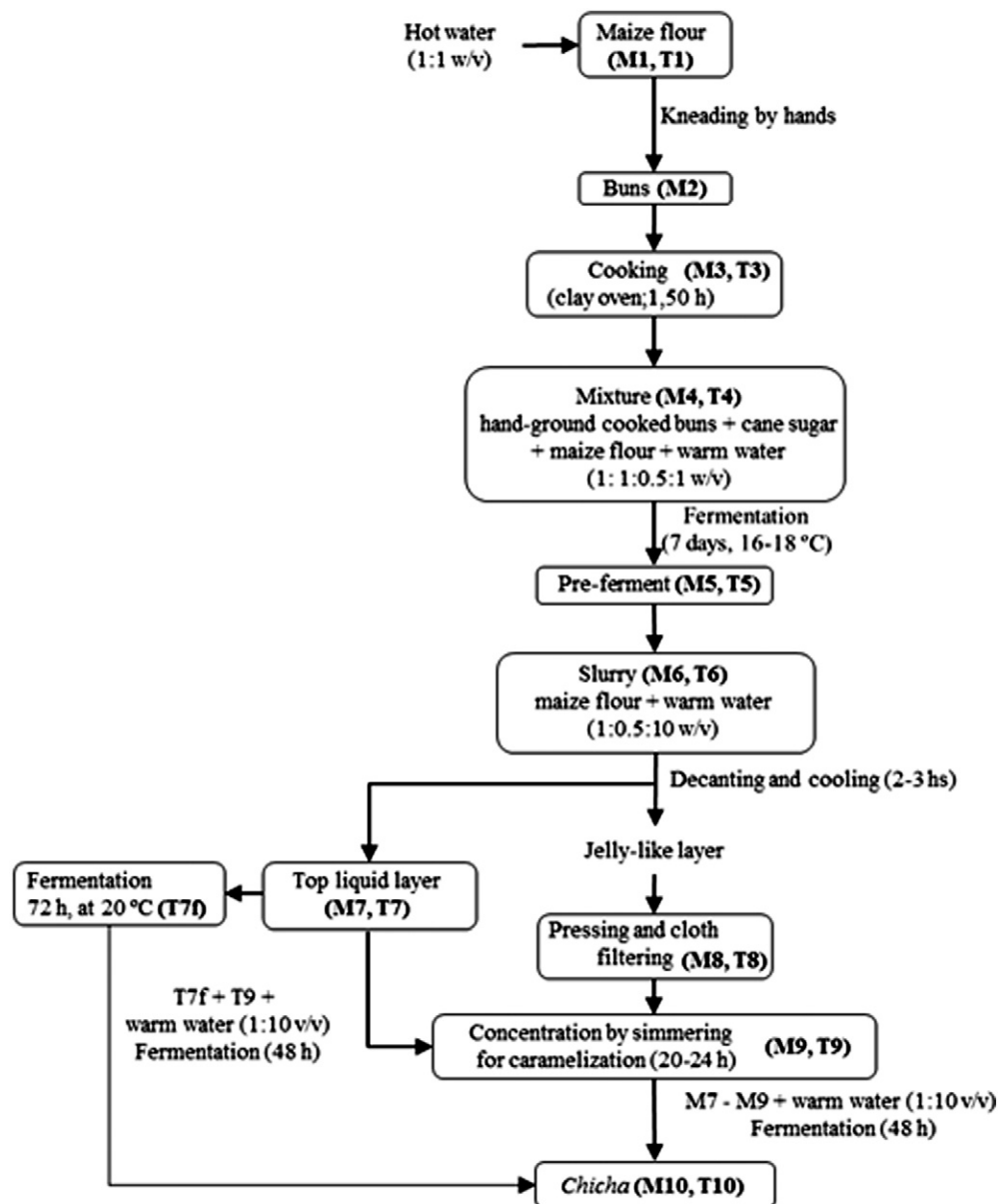
Fig. 1. Flow-chart of *chicha* manufacturing processes corresponding to local productions from Maimará (M) and Tumbaya (T) indicating M1 to M10 and T1 to T10 sampled and analyzed steps.

growth liquid medium containing 20% (v/v) glycerol at −80 °C. DNA from pure cultures was extracted following the guanidium thiocyanate method (Pitcher et al., 1989), spectrophotometrically quantified (Nanodrop 2000™, Thermo Fisher Scientific Inc.) and adjusted to a final concentration of 40 ng/μl in ultra-pure water (Sigma, Madrid, Spain).

2.2.2. PCR-based LAB identification

Identification of isolates into species was approached in three steps. First, genus ascription was based on PCR amplification of the Intergenic Spacer Region (ISR) between the 16S and 23S rRNA genes by comparing electrophoretic profiles with those of reference strains as previously described by Chenoll et al. (2003). As a second step, isolates from each ISR-group (presumptively the same genus), together with reference strains covering different species in the genus, were subjected to RAPD-PCR analysis using universal primer M13 (5′-GAAACAGCTATGACCATG-3′) as described by Aznar and Chenoll (2006). As a third step, species-specific PCR was performed for *Lb. plantarum*/*Lb. pentosus* (Torriani et al., 2001), *Lb. brevis* (Guarneri et al., 2001), *E. faecium* (Dutka-Malen et al., 1995), *E. casseliflavus*/*E. gallinarum* (Layton et al., 2010) and *Lc.*

mesenteroides (Elizaquível et al., 2008). Five microliters of the corresponding amplifications was electrophoretically separated through a 2% agarose gel in TAE buffer at 100 V for 30 min. Gels were stained with Gel Red Nucleic Acid Gel Stain® (Biotium Hayward, Ca, USA) 1:10,000 in 0.1 M NaCl and photographed under UV light. Gel images were recorded using a video camera (Gelprinter Plus, TDI, Madrid, Spain) and stored as TIFF files.

In addition, genomic DNA of selected isolates in each cluster was used for amplification of the almost full-length 16S rRNA gene fragment using the primers 616Valt and 630R as previously described (Chenoll et al., 2003). The 16S rRNA sequences were compared with the RDPII database as described in Section 2.3.3 for species identification.

2.2.3. Cluster analysis of ISR-PCR and RAPD-PCR electrophoretic profiles

Digitized images were converted, normalized, analyzed and combined using the software package BioNumerics 4.61 (Applied Maths, Kortrijk, Belgium). In order to normalize the banding patterns, molecular weight markers were included every seventh track. The levels of similarity between pairs of traces were computed using the Jaccard

Table 1
Sample identification for *chicha* M and *chicha* T manufacturing.

Sampled steps	Chicha M[a]	Chicha T[b]
1	M1. "Criollo" maize flour	T1. "Criollo" maize flour
2	M2. Hand-made buns (maize flour + water)	
3	M3. Cooked buns (clay oven during 1.30 h)	T3. Toasted flour (20 min)
4	M4. Mash (hand-ground cooked buns + cane sugar + maize flour + warm water; 1:1:0.5:1 w/v)	T4. Mash (toasted flour + cane sugar + warm water; 1:0.5:1 w/v)
5	M5. *Mucus* (pre-fermentation during 7 days at 16–18 °C)	T5. *Mucus* (pre-fermentation during 3 days at 20–22 °C)
6	M6. Slurry (pre-ferment + maize flour + warm water; 0.5:1.5:5 w/v)	T6. Slurry (hand mixed pre-ferment + maize flour + warm water; 0.5:2:5 w/v)
7	M7. Top liquid layer (after decanting/cooling of M6 during 2–3 h)	T7. Top liquid layer (after decanting/filtering of T6; repeated 3 times) T7f. Total top liquid layer fermentation for 72 h at 20 °C
8	M8. *Arrope* (jelly-like layer after pressing/cloth filtering)	T8. *Arrope* (jelly-like layer after decanting/cloth filtering)
9	M9. Concentrated *arrope* (simmered for 20–24 h)	T9. Concentrated *arrope* (simmered for 16–18 h)
10	M10. *Chicha* (obtained after 48 h fermentation of M7 + M9 + warm water; 1:10 v/v)	T10. *Chicha* (obtained by mixing T7f + T9 + warm water; 1:10 v/v)

[a] Maimará production.
[b] Tumbaya production.

coefficient for the ISR patterns and the Pearson correlation coefficient that provides similarity based upon densitometry curves for RAPD profiles. Data were clustered using the Unweighted Pair Group Method with Arithmetic (UPGMA) mean algorithm. Identification of profiles was carried out by comparison with a database previously generated (IATA-UVEG) with the aid of the BioNumerics software, containing ISR and RAPD profiles corresponding to 132 reference strains (Chenoll et al., 2007).

2.3. Culture-independent analysis of bacterial populations

2.3.1. Chicha DNA isolation

For DNA isolation, 10 ml of each homogenized sample (Section 2.1.3) was taken from the upper liquid phase and centrifuged at 5000 *g* for 10 min. The pellet was washed three times with 1 ml TE buffer containing 1 mmol l^{-1} EDTA and 10 mmol l^{-1} Tris, pH 8, centrifuged again and resuspended in 200 µl TE buffer. Total DNA was extracted using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total genomic DNA was eluted twice with 50 µl of elution buffer and quantified using the Quant-iT, PicoGreen DNA assay (Invitrogen).

2.3.2. 16S rRNA gene amplification and pyrosequencing

Amplicon library preparation and pyrosequencing were carried out by LifeSequencing Inc. (Valencia, Spain). The DNA isolated from *chicha* samples was used as template for the amplification of the V3–V5 hypervariable region of the bacterial 16S rRNA genes with primer set 357F/926Rb (Sim et al., 2012). Each PCR mixture consisted of 200 µM of each deoxynucleoside triphosphate, 2.5 U of FastStart HiFi polymerase (Roche, Mannheim, Germany), 400 nM of each primer, 4% of 20 g/ml BSA (Sigma, Dorset, UK), 0.5 M betaine (Sigma) and 50 ng of the genomic DNA. Amplification was carried out using a PCR program of 94 °C for 2 min; 30 cycles of 94 °C for 20 s, 50 °C for 30 s and 72 °C for 5 min. After the PCR, amplicon length (651 bp) was determined with a DNA 1 kb Chip using BioAnalizer (Bio-Rad) and quantified with the VersaFluor equipment (Bio-Rad) using a Quant-iT PicoGreen kit (Invitrogen). Amplicons were combined in a single tube in equimolar concentrations. The amplicon pool was purified twice (AMPure XP kit, Agencourt, Takeley, UK) and the cleaned pool was quantified with the VersaFluor equipment (Bio-Rad) using a Quant-iT PicoGreen kit (Invitrogen). Optimal proportion (DNA molecules per beads) for the emulsion PCR (emPCR) was determined with titling. Unidirectional pyrosequencing was carried out on a 454/Roche GS FLX Titanium chemistry system (454 Life Science) following the Roche Amplicon Lib-L protocol.

2.3.3. Bioinformatics and pyrosequencing data analysis

Sequences shorter than 300 bp and those of average quality score lower than 20 were removed using Galaxy server tools (Goecks et al., 2010), while UCHIME algorithm (Edgar, 2011) was applied using RDPII 16S rRNA database as reference to remove chimera sequences. To estimate species richness in each sample, the freeware program aRarefactWin by Holland (http://strata.uga.edu/software/anRareReadme.html) was used and rarefaction curves were obtained. Then, sequences were clustered into OTUs (operational taxonomical units) at 97% sequence similarity to the sequences deposited in RDPII using RDP project tools (Cole et al., 2014). Chao richness, Shannon diversity and Good's coverage were calculated using Mothur v. 1.33.3 software from Michigan University (Schloss et al., 2009). Taxonomic assignment was carried out using the RDP 16S rRNA gene database with a confidence of 90%, those with less than 97% similarity were classified as unidentified.

3. Results

3.1. Physicochemical characterization

During *chicha* manufacturing, a progressive acidification was produced with pH declining from initial values of 5.71 and 5.32 in maize flour to final values of 3.76 and 3.55 for *chicha* M and T, respectively (Table 2). A correlation of pH with titratable acidity was observed, with an increase of lactic acid % from 0.35 to 2.30 in *chicha* M and from 0.25 to 4.30 during *chicha* T manufacturing process. To trace back the substrate utilization during maize fermentation, starchy derivatives such as glucose, maltose and sucrose as well as soluble starch were determined. A decrease in starch, sucrose and maltose content occurred with an increase in glucose concentration throughout both *chicha* production processes. On the other hand, ethanol started to be produced in the early steps of manufacture reaching a final concentration between 9 and 10 g l^{-1} in the beverage.

3.2. Microbial counts at different steps during chicha manufacturing process

Table 2 shows the evolution of microbial counts throughout fermentation of maize for *chicha* production. The trend for the total counts paralleled that of LAB population during both *chicha* manufacturing processes. Total mesophilic counts in PCA varied from 2.4×10^6 and 1.9×10^5 CFU g^{-1} in maize flour used as raw material to 7.2×10^4 and 7.5×10^3 CFU g^{-1} in *chicha* M and T final fermented products, respectively (data not shown). Mesophilic LAB counts in MRS and YGLP media showed similar numbers to those found in PCA (correlation coefficients of 0.95 and 0.96, respectively), suggesting that they are the predominant population. Their initial viable numbers were around 10^6 CFU g^{-1} (*chicha* M) and 10^4 CFU g^{-1} (*chicha* T) and after falling to zero during bun cooking/flour toasting, they reached a maximum of around 10^8 CFU g^{-1} after pre-fermentation. A further reduction of approximately one log cycle in LAB numbers was determined in *arrope*

Table 2
Physicochemical[a] and microbiological[b] parameters during *chicha* M and *chicha* T manufacturing.

Samples	M1/T1	M2	M3/T3	M4/T4	M5/T5	M6/T6	M7/T7	T7f	M8/T8	M9/T9	M10/T10
Chicha M (Maimará)											
pH	5.71	5.95	5.62	5.64	5.07	6.45	7.62		6.67	5.67	3.76
Acidity (g % lactic acid)	0.35	0.30	0.35	1.10	2.20	0.40	0		0.6	1.50	2.30
Glucose (g l^{-1})	2.60	21.6	7.10	29.90	39.70	14.90	2.10		5.30	10.10	12.00
Sucrose (g l^{-1})	5.23	0.02	8.17	16.79	1.13	19.55	8.50		15.07	36.75	0.98
Maltose (g l^{-1})	1.79	4.84	2.57	5.35	1.13	3.78	1.10		0.05	1.52	0.11
Starch (g 100 g^{-1})	54.52	36.04	61.20	24.75	29.12	7.50	1.83		4.55	16.71	2.72
Ethanol (g l^{-1})	0	0.34	0	0.24	1.32	0.24	0.15		0.34	0.14	9.45
Total counts	2.4×10^6	3.0×10^8	0	6.0×10^4	1.5×10^8	8.4×10^8	2.9×10^7		9.7×10^7	5.2×10^7	7.2×10^4
LAB (YGLP)	2.7×10^6	2.9×10^7	0	5.5×10^4	6.8×10^7	2.8×10^8	2.0×10^7		4.0×10^7	5.0×10^7	2.2×10^5
LAB (MRS)	2.0×10^6	5.5×10^8	0	4.5×10^4	4.5×10^7	1.2×10^9	4.0×10^7		1.5×10^8	6.7×10^7	5.0×10^4
Total yeast and molds	$.1 \times 10^6$	$.0 \times 10^3$	0	$.1 \times 10^4$	$.0 \times 10^4$	$.0 \times 10^2$	$.0 \times 10^2$		$.0 \times 10^2$	10^2	$<10^2$
Total coliforms	1.5×10^5	8.0×10^7	0	5.5×10^4	–	–	–		–	–	–
Chicha T (Tumbaya)											
pH	5.32		4.87	5.88	5.19	4.45	4.50	3.76	5.10	4.93	3.56
Acidity (g % lactic acid)	0.25		0.20	0.50	0.9	3.45	0.80	1.45	1.50	1.05	4.30
Glucose (g l^{-1})	1.08		0.44	0.30	3.24	17.48	5.61	4.82	3.32	1.32	14.16
Sucrose (g l^{-1})	2.60		0.01	58.50	53.10	10.07	1.63	6.05	0.05	0.03	0.02
Maltose (g l^{-1})	0.94		0.02	1.18	0.03	1.80	0.07	0.01	1.17	0.11	0.86
Starch (g 100 g^{-1})	73.11		42.63	18.50	22.60	29.30	5.45	0.95	1.52	0.92	2.42
Ethanol (g l^{-1})	0		0	0.32	0.75	0.65	0.21	0.37	0.48	0.27	9.98
Total counts	1.9×10^5		0	2.5×10^4	2.3×10^8	2.2×10^8	3.7×10^8	4.8×10^2	4.3×10^7	3.5×10^7	7.5×10^3
LAB (YGLP)	3.4×10^4		0	1.4×10^4	2.4×10^8	2.2×10^8	3.6×10^8	8.3×10^2	3.9×10^7	4.0×10^7	2.3×10^3
LAB (MRS)	3.2×10^4		0	5.5×10^3	2.2×10^8	2.4×10^8	2.0×10^8	3.9×10^2	5.1×10^7	4.6×10^7	9.7×10^2
Total yeast and molds	1.1×10^6		0	6.0×10^4	1.2×10^4	3.0×10^3	6.0×10^2	1.0×10^2	1.0×10^2	$<10^2$	$<10^2$
Total coliforms	1.1×10^6		0	2.4×10^3	1.0×10^4	9.5×10^3	1.0×10^1	–	–	–	–

[a] Values are means of duplicate measurements.
[b] Values of microbial counts are means of triplicates. Standard deviations were always lower than 10% of the means.

(M9). When this concentrated fraction was mixed with the top liquid layer (M7), diluted with water and allowed to ferment for 48 h the resultant final product showed LAB counts of 10^4–10^5 CFU g^{-1} (*chicha* M). Regarding *chicha* T, although there is a decrease of LAB in the liquid layer (T7f), when this fraction was mixed with concentrated *arrope* a LAB load of 10^7 CFU g^{-1} was found; a final beverage with LAB counts around 10^3 CFU g^{-1} was then obtained. Counts in MRS-S and MRS-M media were similar (data not shown). No isolates able to hydrolyze soluble starch on plates (transparent halo around colonies) were observed. For both *chicha* productions total coliform counts were maximal in the raw material (maize flour) and hand kneading/mixing process, but they disappeared after the decantation/separation of top liquid layer step (M7 and T7). Yeast and mold counts showed higher variability ranging from 10^6 to $<10^2$ CFU g^{-1} throughout the *chicha* manufacturing process.

3.3. Identification of LAB isolates using molecular techniques

A total of 146 colonies from *chicha* 1 and 246 from *chicha* 2 recovered from MRS and YGLP plates were considered as presumptive LAB because they were Gram-positive and catalase-negative. Analysis of LAB isolates approached by ISR-PCR fingerprinting of whole genomes and RAPD-PCR is shown in Supplementary Figs. 1 and 2. First, 16S–23S ISR amplification yielded one to five bands of molecular sizes ranging from 300 to 1000 bp corresponding to the genera: *Leuconostoc*, *Lactobacillus/Pediococcus*, *Weissella*, *Lactococcus* and *Enterococcus*. RAPD-PCR using M13 phage-based universal primer (RAPD-M13) was further approached for genotypic differentiation. Clustering analysis of the combined ISR and RAPD-M13 profiles was performed individually for each genus, including food isolates and reference strains for each *chicha* production. Ascription of food isolates into species was based on the clusters derived from the combined analysis of RAPD-M13 and ISR profiles, assessed by 16S rRNA gene sequencing of 1 to 3 representative strains per profile/cluster. Twenty-one 16S rRNA gene sequences

were analyzed from *chicha* M and nineteen from *chicha* T, showing similarity levels of 99% when compared with public sequences of LAB species. In addition, when available, species identification was confirmed by PCR amplification using species-specific primers.

During *chicha* M production, recovered *Leuconostoc* isolates (57) belonged to the species *Lc. mesenteroides* and *Lc. lactis*; *Lactobacillus* (34) were identified as *Lb. plantarum* and *Lb. brevis* and all *Weissella* isolates (28) belonged to the species *W. viridescens*. On the other hand, for *chicha* T production, *Enterococcus* isolates (194) belonged mainly to *E. faecium* (187) species and a minor proportion to *Enterococcus durans* (7); all *Leuconostoc* isolates (50) corresponded to the species *Lc. mesenteroides*; *Pediococcus acidilactici* isolates (2) were identified based on 16S rRNA gene sequences.

3.4. Distribution of LAB species along the chicha manufacturing process

Distribution of LAB species along the *chicha* manufacturing process and the frequency of occurrence at each step of *chicha* M (Maimará) and *chicha* T (Tumbaya) productions are summarized in Table 3. During *chicha* M manufacturing, *Leuconostoc* and *Lactobacillus* species represented 39% and 23% of total isolates, respectively; in a lesser extent, *W. viridescens* 20% and *Enterococcus* species 18% were also identified. From a dynamic point of view, the presence of *Lactobacillus* was detected mostly after the pre-fermentation stage (M5), and jelly-like layer (M8); the greatest *Leuconostoc* population was evidenced in the top-liquid and jelly-like layers (M7, M8). *W. viridescens* was predominantly present when maize flour was added (M2 and M6 samples), while *Enterococcus* species were detected in "criollo" maize flour (M1) and the final product (M10). On the other hand, during *chicha* T production, *Enterococcus* species were by far, the most represented LAB population (79%), *E. faecium* being present in all samples with the exception of the final product (T10). Similarly, as the second LAB population, *Leuconostoc* (20%) were detected throughout the manufacturing process whereas *P. acidilactici* (1%) were isolated in T1 and T7f samples.

Table 3
Distribution of species along the *chicha* manufacturing process.

Sampled steps																					Total	
	1		2		4		5		6		7			8		9		10			M	T
Chicha production	M1	T1	M2	T2	M4	T4	M5	T5	M6	T6	M7	T7	T7f	M8	T8	M9	T9	M10	T10		M	T
Number of isolates	17	38	21	0	0	30	21	25	18	23	18	28	39	18	28	18	34	15	1		146 (%)	246 (%)
Leuconostoc	–	10	8	–	–	–	1	1	–	16	18	–	2	18	–	6	9	6	1		57 (39)	48 (20)
Lc. lactis	–	–	–	–	–	–	–	–	–	–	18	–	–	5	–	3	–	4	–		30 (21)	–
Lc. mesenteroides	–	10	8	–	–	–	1	1	–	16	–	–	2	13	–	3	9	2	1		27 (18)	48 (20)
Lactobacillus	–	–	2	–	–	–	20	20	–	–	–	–	–	–	–	12	–	–	–		34 (23)	–
Lb. plantarum	–	–	2	–	–	–	19	19	–	–	–	–	–	–	–	12	–	–	–		33 (22)	–
Lb. brevis	–	–	–	–	–	–	1	1	–	–	–	–	–	–	–	–	–	–	–		1 (0.7)	–
Weissella	–	–	10	–	–	–	–	–	18	–	–	–	–	–	–	–	–	–	–		28 (20)	–
W. viridescens	–	–	10	–	–	–	–	–	18	–	–	–	–	–	–	–	–	–	–		28 (20)	–
Enterococcus	17	27	1	–	–	30	–	–	–	7	–	28	36	–	28	–	25	9	–		27 (18)	196 (80)
E. casseliflavus	17		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–		17 (12)	–
E. faecium	–	27	–	–	–	23	–	–	–	7	–	28	36	–	28	–	25	9	–		9 (6)	189 (77)
E. mundtii	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–		1 (0.7)	–
E. durans	–	–	–	–	–	7	–	–	–	–	–	–	–	–	–	–	–	–	–		–	7 (3)
Pediococcus	–	1	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–		–	2 (1)
P. acidilactici	–	1	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–		–	2 (1)

[a] T3 and S3 did not render LAB isolates; M: Maimará production; T: Tumbaya production.

3.5. Identification of the bacterial community as determined by HTS

Identification of the bacterial populations associated to the *chicha* manufacturing process was pursued by high-throughput 454 pyrosequencing. Partial 16S rRNA gene sequencing was obtained from DNA directly extracted from seventeen *chicha* samples, nine from *chicha* M and eight from *chicha* T. Samples corresponding to post heat-treatments (M3, T3) and *chicha* T slurry (T6) did not yield good quality amplifiable DNA. After quality control, 133,305 reads with an average length of 541 bp were obtained and analyzed (73,174 for *chicha* M and 59,591 for *chicha* T). Reads were distributed among samples as reported in Table 4. Samples from *chicha* M were covered by an average of 9132 reads/sample (except for M10), while for *chicha* T the average coverage was 7448 reads/sample. The rarefaction analysis and the diversity indexes indicated that there was satisfactory coverage of the diversity within both *chicha* productions. The highest diversity and richness was associated with M4, M10, T1, T4 and T10. When the relative abundance of the taxonomic levels was calculated at phylum level, it was observed that *Firmicutes* and *Cyanobacteria* predominated in both *chicha* productions. Since maize is the main component in *chicha*, it was assumed that *Cyanobacteria* sequences correspond to maize chloroplasts and therefore they were not included in the analysis (Table 4). Fig. 2 shows the distribution of LAB, Families (2a) and Genera (2b), along *chicha* M and T manufacturing processes. As it can be observed that LAB were minority in samples M1, M4 and M7 and T1 and T4 corresponding to the addition of maize flour or cane sugar; LAB became majority following the fermentation steps. Sequence assignment at genus level showed that *Enterococcus*, *Lactococcus*, *Streptococcus*, *Weissella*, *Leuconostoc* and *Lactobacillus* were detected in both productions, while *Pediococcus* were only identified in *chicha* M. Although *Enterococcus* were present in both productions, this LAB genus was predominant in *chicha* T. Both "criollo" maize flours (M1, T1) displayed the lowest diversity with only *Enterococcus* and *Lactobacillus* in *chicha* M, and only *Enterococcus* and *Weissella* in *chicha* T. On the other hand, the greatest diversity at genus level during *chicha* M manufacture was found in samples M4 and M10 with six genera represented (*Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus* and *Weissella*). In *chicha* T the greatest diversity was found in T4, which included all LAB identified genera, followed by T7f and T10 samples where with the exception of *Streptococcus*, all other genera were present.

To analyze the bacterial community during the *chicha* M and T manufacturing at species level, a heat-map representing the relative

Table 4
Number of sequences analyzed, observed diversity richness (OTUs), estimated OTU richness (Chao 1), diversity index (Shannon), and estimated sample coverage for 16S rRNA amplicons from *chicha* M and T productions.

Sample	No. sequences		TUs	Chao richness	Shannon diversity	Goods coverage
	A	B				
M1	9156	880	28	337.3 (243.54; 507.13)	2.36 (2.22; 2.5)	89.54%
M2	10,471	10,322	323	1544.1 (1113.1; 2210.3)	0.96 (0.92; 0.99)	97.46%
M4	8846	1857	332	1217.5 (921.6; 1661.9)	3.5 (3.4; 3.7)	82.61%
M5	6418	6240	286	1504 (1040.5; 2252.3)	0.89 (0.84; 0.95)	96.28%
M6	6813	6813	83	321.3 (193.9; 595.1)	0.27 (0.24; 0.3)	99.03%
M7	9427	9398	274	1545.2 (1048.3; 2361)	0.95 (0.91; 0.99)	97.59%
M8	11,628	11,139	144	749 (463.9; 1287.8)	0.16 (0.14; 0.18)	98.91%
M9	10,300	10,300	166	831 (528.9; 1394.7)	0.98 (0.95; 1.01)	98.71%
M10	655	645	191	422.6 (332.7; 569.6)	4.24 (4.12; 4.36)	80.43%
T1	8034	277	75	181.2 (124.3; 303.9)	3.23 (3.05; 3.41)	81.58%
T4	2746	2579	456	1548.5 (1232.7; 1992.6)	3.2 (3.1; 3.3)	86.66%
T5	11,584	10,876	172	847.2 (543.9; 1397.8)	0.81 (0.78; 0.84)	98.73%
T7	9916	9771	32	438 (230.3; 863.1)	0.03 (0.02; 0.04)	99.7%
T7f	7453	7452	104	261.6 (180; 430.6)	0.53 (0.5; 0.57)	99.16%
T8	8146	8141	50	155.6 (89; 335.8)	0.32 (0.29; 0.34)	99.59%
T9	7951	7951	47	159.2 (88.7; 348.7)	0.27 (0.25; 0.29)	99.57%
T10	3761	3761	41	351.8 (256.2; 526.5)	1.75 (1.69; 1.81)	97.6%

A, total number of sequences including *Cyanobacteria*. B, number of sequences excluding *Cyanobacteria*.
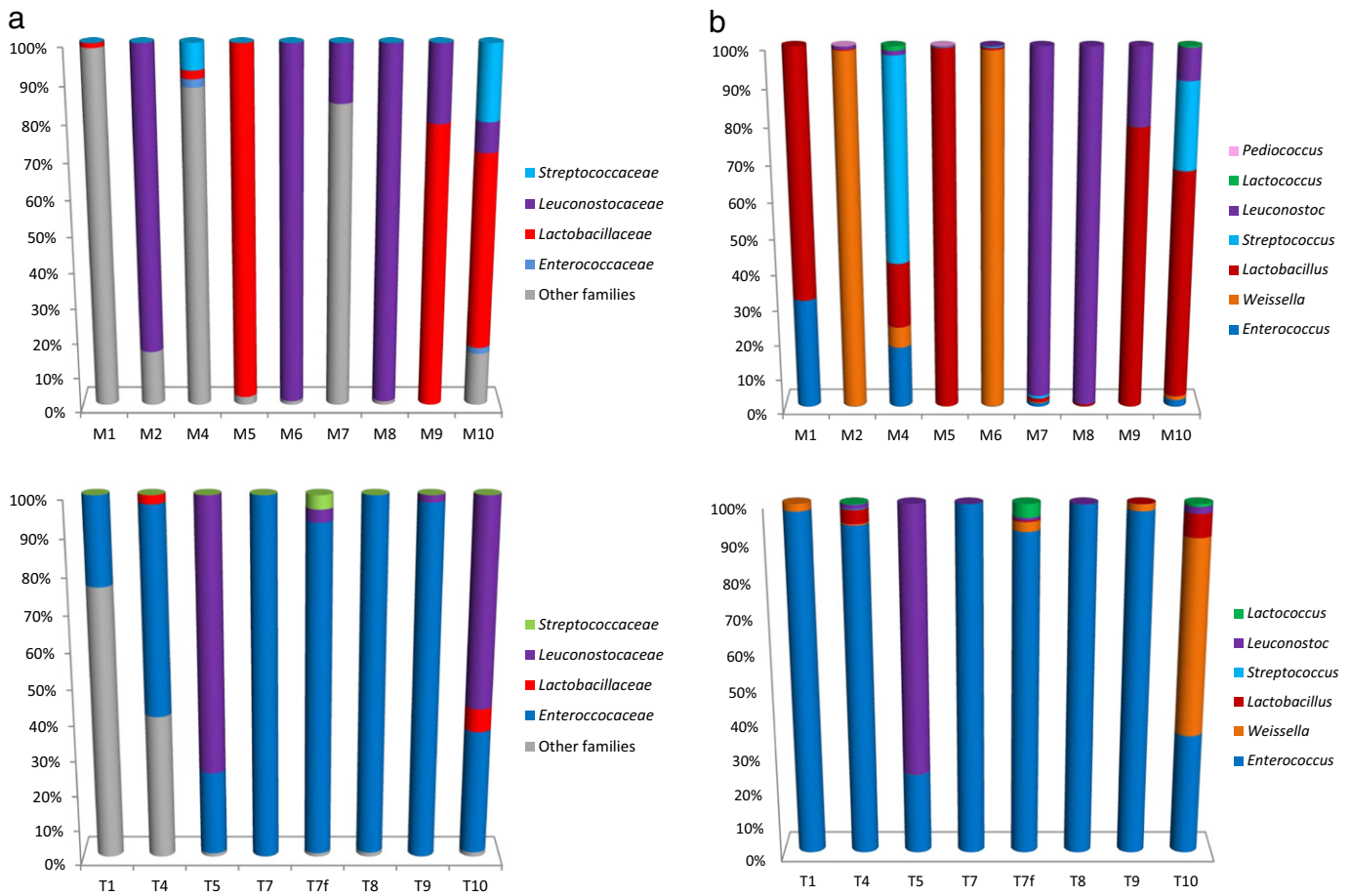
Fig. 2. Identification of bacterial populations in *chicha* productions from Maimará (M) and Tumbaya (T) derived from HTS analysis. Relative abundance of LAB and other families (a) and distribution of LAB in genera (b) are shown. Samples corresponding to post heat-treatments (M3, T3) and *chicha* T slurry (T6) did not yield good quality amplifiable DNA and were excluded.

abundance of the OTUs with an abundance above 0.1% in at least one sample was constructed (Fig. 3). Forty-four OTUs belonging to the LAB group were considered; 39 of them were found in *chicha* M and 29 in *chicha* T.

The greatest species diversity was found in *Lactobacillus*, with 25 identified OTUs, 13 of them present exclusively in *chicha* M. It was followed by *Enterococcus*, with 8 species identified, all of them present during the manufacturing process of *chicha* T. Four species were identified belonging to the genera *Leuconostoc* and *Weissella*, two to *Streptococcus* and one to *Lactococcus*. The most abundant species during *chicha* M manufacturing process was *W. viridescens* in samples M2 (83.6%) and M6 (98.75%); *Lc. lactis* in M8 (85.82%), *Lb. plantarum* in M9 (73.52%) and *Lactobacillus rossiae* in M5 (63.55%). In *chicha* T, *Enterococcus hirae* predominated in samples T7 (94.75%), T7f (86.1%), T8 (93.04%) and T9 (92.2%), followed by *Lc. mesenteroides* in sample T5 (75.5%), *Weissella confusa* in T10 (54.81%) and *E. faecium* in T4 (46.4%).

4. Discussion

Although there is no universal strategy to investigate the microbial biodiversity of complex matrices, the use of more than one methodology might provide a better global overview of the microbial composition. This approach is particularly important for traditional foods/beverages that have not been deeply investigated yet since bacterial species with valuable properties can be lost when using the classical culture based approach. Evidences on this fact are increasing as new cultivation independent molecular methodologies are being applied (Cruciata et al., 2014; Gulitz et al., 2013). In this sense, food microbiology has recently

benefited from the advances in molecular biology and adopted novel strategies to detect, identify, and monitor microbes in food. HTS approaches after direct nucleic acid extraction from samples have become an essential tool for in-depth analysis of the microbial diversity in natural ecosystems (Ercolini, 2013; Petrosino et al., 2009). HTS provides a snapshot of the bacterial population while traditional culturing techniques allow the recovery of potentially relevant strains. In the present study the LAB populations associated to *chicha* production were analyzed both by pyrosequencing which provided information about the bacterial community, and culturing for strain recovery. In addition, information from other bacterial groups was retrieved, either by plate counts or pyrosequencing, and it has been reported to enhance the description of the ecosystem. For comparative purposes, microbiological and physicochemical parameters were determined. Both M and T productions showed high LAB counts (approx. 10^8 CFU/ml) during the manufacturing process paralleling the pH decrease. This behavior resembles those found for other traditionally fermented cereal-based products, except for the final LAB values (10^3–10^5 CFU/ml) that were consistently lower in *chicha* than other reported traditional alcoholic beverages (Botes et al., 2007). As LAB are resistant to low pH, dilution (with warm water) carried out towards the end of the *chicha* processing as well as the effect of increased ethanol concentration may explain the lower final LAB counts. Besides, the reduction in the content of disaccharides through the manufacturing process suggests that LAB growth was supported by maltose and sucrose which are among the main soluble sugars of maize kernels (1–3%) (FAO, 1992). Therefore, enzymatic processes other than those produced by LAB may occur in order to provide low-molecular weight malto-oligosaccharides supporting the growth of
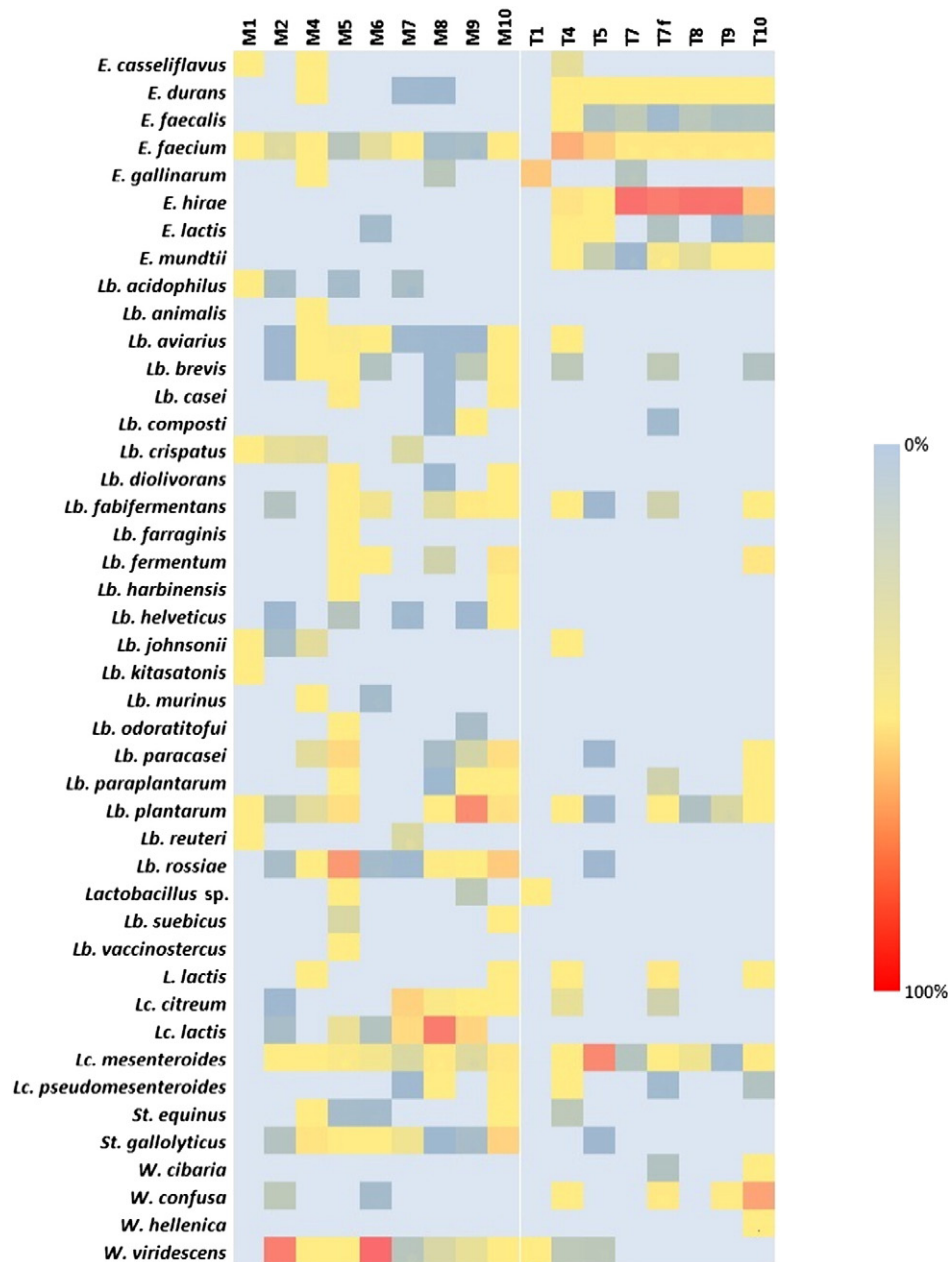
Fig. 3. Heat-map showing LAB species abundance and distribution during the manufacturing processes of *chicha* M and T. Species accounting for more than 0.1% are represented.

non-amylolytic microorganisms (Díaz-Ruiz et al., 2003). Accordingly, since breakdown of large macromolecules such as starch is higher in deeper layers with less oxygen, this process probably occurs in the residual layer before decanting, and although it was discarded, microorganisms in the upper layers could benefit from the released maltose. In addition, the presence of yeast throughout the process confirmed the final alcohol content recorded in this study. Concerning coliforms, despite the fact that a high level was registered in the buns probably because of the manipulation, after the pre-fermentation step, they were no further recovered. The outgrowth of LAB during fermentation may explain coliform decrease.

Regarding LAB populations, both approaches evidenced differences in species composition during *chicha* M and T productions that may be associated with the different manufacturing practices and raw material used by the local producers. In the present study, *Lactobacillus* and *Leuconostoc* dominated in *chicha* M and *Enterococcus* in *chicha* T. The predominance of *Lactobacillus* in maize-based spontaneous fermentations has been described (Abriouel et al., 2006; De Vuyst et al., 2014;

Schoustra et al., 2013) while the presence of *Leuconostoc* has been found as secondary population in other traditional vegetable fermented products (De Vuyst et al., 2014; Jung et al., 2011; Schoustra et al., 2013).

Among the majority species, *Lb. plantarum* was present after concentration and simmering of the jelly-like layer (M9) suggesting its adaptation to the changing conditions encountered along *chicha* manufacturing, in agreement to a highly adapted carbohydrate metabolism, and the lifestyle adaptation region found in its genome (Kleerebezem et al., 2003). This fact is in line with the high intraspecific diversity observed in *Lb. plantarum* (Supplementary Fig. 1) as previously pointed out by Molenaar et al. (2005).

Only two *Leuconostoc* species, *Lc. mesenteroides* and *Lc. lactis* were recovered by culture out of four species revealed by HTS. In *chicha* M, *Lc. mesenteroides* and *Lc. lactis* were identified by culturing and HTS; the former was also the most representative during *chicha* T whereas in *chicha* M, *Lc. lactis* accounted for the greatest incidence as determined by HTS. *Lc. mesenteroides* was often reported in vegetable products such as sourdoughs (De Vuyst et al., 2014), alcoholic fermented beverages

(Escalante et al., 2008) and other traditional vegetable fermented products (Amoa-Awua et al., 2005; Jung et al., 2011).

Leuconostocaceae were also represented by *Weissella*; among the detected species in both *chicha* beverages, *W. viridescens* was recovered by HTS and culturing and exhibited the greatest incidence in *chicha* M while *W. confusa* was detected only by HTS and was the most abundant species in final *chicha* T beverage. Despite *Weissella* species being commonly found in a variety of habitats such as soil, fresh and fermented vegetables and meat products (Justé et al., 2014; Koort et al., 2006; Magnusson et al., 2002), the presence of *W. viridescens* in *chicha* M suggests a cross-contamination during grain milling since it is usually recovered from meat products (Koort et al., 2006). On the other hand, *W. confusa* was also retrieved among the predominant LAB from African fermented porridge and beverages (Lei and Jakobsen, 2004; Vieira-Dalode et al., 2007).

Enterococci represented the largest LAB population in *chicha* T, with *E. hirae* and *E. faecium* as the predominant species. *E. faecium* was recovered by culturing together with *E. casseliflavus*, *E. durans*, and *E. mundtii*, whereas *E. faecalis*, *E. gallinarum*, *E. hirae* and *Enterococcus lactis* were also revealed when HTS was applied. Enterococci may represent contaminants from the environment and grain manipulation during milling; in fact they have been reported among the mesophilic bacteria isolated from water, soils, cereal flours and other vegetable materials (Abriouel et al., 2008; De Vuyst and Neysens, 2005; Van der Meulen et al., 2007). In particular, *E. casseliflavus* and *E. mundtii* are known to be plant-associated bacteria (Devriese et al., 2006) while *E. hirae* was reported from bovine rumen as well as river and wastewater (Abriouel et al., 2008; Arokiyaraj et al., 2014). Moreover, the presence of *E. faecium* may be linked to the added warm water during manufacturing; besides being frequently water borne contaminants, enterococci are among the most thermotolerant of the nonsporulating bacteria. The ability to cope with harsh environmental conditions such as the presence of ethanol was also reported (Capozzi et al., 2011).

In traditional vegetable fermented products, the type and quality (enzymatic, microbiological and nutritional) of the cereal flour as well as process technology play a key role in establishing stable microbial consortia. Species and/or strains adapted to the manufacturing environment regarding nutrient availability (amino acids, fatty acids, minerals, vitamins and other growth factors), endogenous enzymes, and autochthonous LAB and yeasts will thrive in the ecosystem. Therefore, among other mechanisms, competitiveness may explain the apparent prevalence of certain LAB species and/or strains (De Vuyst et al., 2014).

In the present study HTS was used for the first time to analyze diversity and evolution of bacterial populations associated to the *chicha* production. The fine inventory achieved by HTS revealed minor OTUs occurring during both maize-based *chicha* beverage manufactures showing similarities and differences in the bacterial community of both geographically separated productions. Species identified and their dynamics along manufacturing clearly reflected the changing environmental conditions encompassing the different process operations during both *chicha* productions. The results of this work extend the knowledge about LAB participating in the *chicha* fermentation process pointing to the species to be further investigated as functional strains and/or potential starters.

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