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Yeast diversity during the fermentation of Andean *chicha*: A comparison of high-throughput sequencing and culture-dependent approaches.

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ABSTRACT

Diversity and dynamics of yeasts associated with the fermentation of Argentinian maize-based beverage *chicha* was investigated. Samples taken at different stages from two *chicha* productions were analyzed by culture-dependent and culture-independent methods. Five hundred and ninety six yeasts were isolated by classical microbiological methods and 16 species identified by RFLPs and sequencing of D1/D2 26S rRNA gene. Genetic typing of isolates from the dominant species, *Saccharomyces cerevisiae*, by PCR of delta elements revealed up to 42 different patterns. High-throughput sequencing (HTS) of D1/D2 26S rRNA gene amplicons from *chicha* samples detected more than one hundred yeast species and almost fifty filamentous fungi taxa. Analysis of the data revealed that yeasts dominated the fermentation, although, a significant percentage of filamentous fungi appeared in the first step of the process. Statistical analysis of results showed that very few taxa were represented by more than 1% of the reads per sample at any step of the process. *S. cerevisiae* represented more than 90% of the reads in the fermentative samples. Other yeast species dominated the pre-fermentative steps and abounded in fermented samples when *S. cerevisiae* was in percentages below 90%. Most yeasts species detected by pyrosequencing were not recovered by cultivation. In contrast, the cultivation-based methodology detected very few yeast taxa, and most of them corresponded with very few reads in the pyrosequencing analysis.

1. Introduction

Fermentation is one of the oldest methods of food preservation. Traditional fermentation of food staples serves as a substitute where refrigeration or other means of preservation are not available for the safekeeping of food (Holzapfel, 2002). Fermented products play also an important role in rural life contributing to income generation at small scale business. Moreover, fermented products are valued as major dietary constituents because of their highly appreciated sensory attributes and added value (Steinkraus, 2002). In South America, Andean grains have been cultivated for thousands of years since pre-Colombian times, maize being the most representative and consumed of the cereals (Perry et al., 2006). Moreover, Andean cereals and particularly maize are the substrates for production of several traditional fermented foods and beverages important for the culture and traditions of Andean population (Wacher et al., 2000; Blandino et al., 2003; Osorio-Cadavid et al., 2008; Faria-Oliveira et al., 2015). Among them, under the name of *chicha*, a variety of homemade fermented beverages derived from different plant materials such as maize, rice, cassava, peanut or carob, are produced. These traditional beverages are mainly consumed by the native population during religious and agricultural festivities as well as during family and social events (Lorence-Quiñones et al., 1999; Delibes and Barragán, 2008). Maize based *chicha* is a clear, yellowish, effervescent, alcoholic (2–12% v/v) beverage consumed since pre-Hispanic times using primarily local maize varieties. Traditionally, the production of *chicha* involved saliva as inoculum, which served as the source of amylase to convert starch to fermentable sugars (Escobar et al., 1996). Nowadays, other alternatives for amylase production are malting (germination) of maize kernels (*chicha de jora*) or a pre-fermentation step involving heated water during the manufacture

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process (Lorence-Quiñones et al., 1999).

The fermentation of traditional cereal-based foods and beverages is a spontaneous process commonly conducted by wild microbiota involving bacteria, yeast and fungi (Blandino et al., 2003; Lacerda Ramos et al., 2010). Yeasts have been found in several indigenous fermented products from different countries (Gotcheva et al., 2000; Osorio-Cadavid et al., 2008; Santos et al., 2012; Greppi et al., 2013). However, yeast population dynamics have been poorly studied and their role during the fermentation process is therefore not well understood. In the case of *chicha*, previous studies using traditional isolation methodologies have revealed lactic acid bacteria and yeasts as the dominant populations (Escobar et al., 1996; Steinkraus, 1996; Faria-Oliveira et al., 2015). In case of bacteria, Puerari et al. (2015) and Elizaquivel et al. (2015) applying culture dependent and independent methods found a codominance of LAB (lactic acid bacteria) genera *Lactobacillus*, *Enterococcus* and *Leuconostoc*. On the other hand, yeasts have been solely investigated by conventional cultural methods, which revealed *S. cerevisiae* as the dominant species in the fermentative process (Steinkraus, 1996; Blandino et al., 2003; Vallejo et al., 2013). In recent years, the study of microbial diversity in food has radically changed with the application of next-generation sequencing (NGS) methods to mixed DNA extractions obtained directly from food samples. Among the strengths of the NGS methods is the generation of thousands of sequences per sample yielding a quantitative and representative in-depth picture of the microbial diversity present in the sample, and the possibility to sequence several samples simultaneously in multiplexing approaches using sample-specific bar codes depending on the desired level of sample coverage (Ercolini, 2013). Although NGS has been successfully applied to analyze the bacterial communities of several traditional fermentations (Van Hijum et al., 2012) its application to unveil yeast diversity is still very limited.

The aim of this study was to obtain a comprehensive picture of the diversity and dynamics of yeasts associated with the fermentation of the maize beverage *chicha* produced in Northwest region of Argentina. For this purpose, samples taken at different stages from two *chicha* productions were analyzed by culture-dependent and culture-independent methods.

2. Materials and methods

2.1. Chicha manufacturing process and sample collection

A total of twenty samples from two productions of *chicha* prepared by local producers from Maimará and Tumbaya villages (Quebrada de Humahuaca region) in Northwest of Argentina were analyzed. The samples were aseptically collected in sterile bottles and maintained under refrigeration until handling in the laboratory. Fig. 1 shows the flow-chart for *chicha* manufacturing process. *Chicha* was prepared using "*criollo*" maize flour which is an ancient variety cultivated in this Andean region. Maize flour was prepared in buns by soaking the maize in warm water (Maimará *chicha*) or directly toasted (Tumbaya *chicha*) on a tray in a rudimentary clay oven. A mash was prepared by mixing the toasted buns or flour with cane sugar, warm water (Tumbaya *chicha*) and additional *criollo* maize flour (Maimará *chicha*). Fermentation of this mash was allowed for 3–7 days depending on the room temperature. The fermented mash, "*mucus*", was supplemented with commercial maize flour and warm water to obtain a slurry. This slurry was thoroughly mixed and allowed to cool and precipitate for 2–3 h. The slurry was separated in three layers, the top liquid layer, "*chuia*", the jelly-like middle layer and the bottom solid layer, "anchi". The jelly-like middle layer was pressed and cloth filtered. The filtered liquid was concentrated and caramelized by simmering producing the "*arrope*". "*Chuia*" and "arrope" were pooled and diluted with water to constitute the Maimara *chicha*. In case of the Tumbaya *chicha* the mix of "*arrope*" and "*chuia*" was allowed to ferment for 2 days. Tumbaya *chicha* was then stored at low temperature for 7–10 days until bubbling ceased and was ready to consumption.

2.2. Physicochemical characterization of chicha

pH measurements were taken using a digital pHmeter (Model PT-10, Sartorius AG, Germany). Titratable acidity, expressed as lactic acid %, was analyzedon10 ml of each sample using NaOH0.1 N and phenolphthalein as indicator. Starch content was determined using the method described by Tovar et al. (1990). Ethanol concentration was determined using the kit "Ethanol UV-method" (Roche, R-Biopharm, Boehringer Mannheim, Germany).

2.3. Yeast enumeration and isolation

For enumeration and isolation of yeasts during *chicha* production, 5 g of each sample was resuspended in 45 ml of sterile saline solution (0.9%) and homogenized three times for 30 s using a Pulsifier (MicrogenBioproducts, Surrey, UK). Yeast counts were carried out using a decimal serial dilution in saline solution and plating onto culture media. Aliquots of 0.1 ml were plated onto malt extract agar (MEA) (malt extract 5% and agar 2%), GPYA (glucose 2%, peptone 0.5%, yeast extract 0.5% and agar 2%), APYA (same as GPYA but glucose replaced by soluble starch) and SPYA (same as GPYA but glucose replaced by sucrose) and incubated at 28 °C and 12 °C for 3–5 days. Plating on MEA and GPYA was done in triplicate for estimation of CFU/g, whereas APYA and SPYA were used as additional yeast isolation media and plating was done in duplicate. All media were supplemented with chloramphenicol (100 mg/l) and ampicillin (50 mg/l) to inhibit bacterial growth. After incubation a random number of colonies representing all morphologies was selected. Isolates were purified by streak plating on GPYA. The purified isolates were maintained at - 80 °C in 15% (v/v) glycerol.

2.4. Qualitative screening of yeast species for amylolytic activity

Yeast isolates were spotted on YPD (yeast extract 1%, peptone 2% and agar 2%) containing 1% soluble starch as sole carbon source (Strauss et al., 2001). Plates were incubated for 6 days at 28 °C before being flooded with Lugol's iodine solution. Production of amylase was indicated by presence of a clear halo around the amylolytic colonies.

2.5. DNA extraction and molecular identification of yeast isolates

Yeast cells were grown in GPY broth at 28 °C. Total genomic DNA was extracted according to Querol et al. (1992). Tentative identification of the isolates was performed by PCR-RFLP of the ITS1-5.8S-ITS2 rDNA region following the methodology described by Esteve-Zarzoso et al. (1999) by comparison with the patterns deposited in the Yeast-id database (www.yeast-id.com). Amplification of D1/D2 domains of the 26S rRNA gene was achieved using PCR primers NL1 (5-GCATATCAATAAGCGGAGGAAAAG-3) and NL4 (5-GGTCCGTGTTTCAAGACGG-3). PCR reactions were carried out under the same conditions used to amplify theITS1-5.8S-ITS2 rDNA region. PCR products were purified using the High Pure PCR Purification kit (Roche, Switzerland) following the manufacturer's instructions. DNA sequencing was conducted using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA) in an Applied Biosystems (Model 310) automatic DNA sequencer. Sequences were edited using MEGA6 (Tamura et al., 2013) and

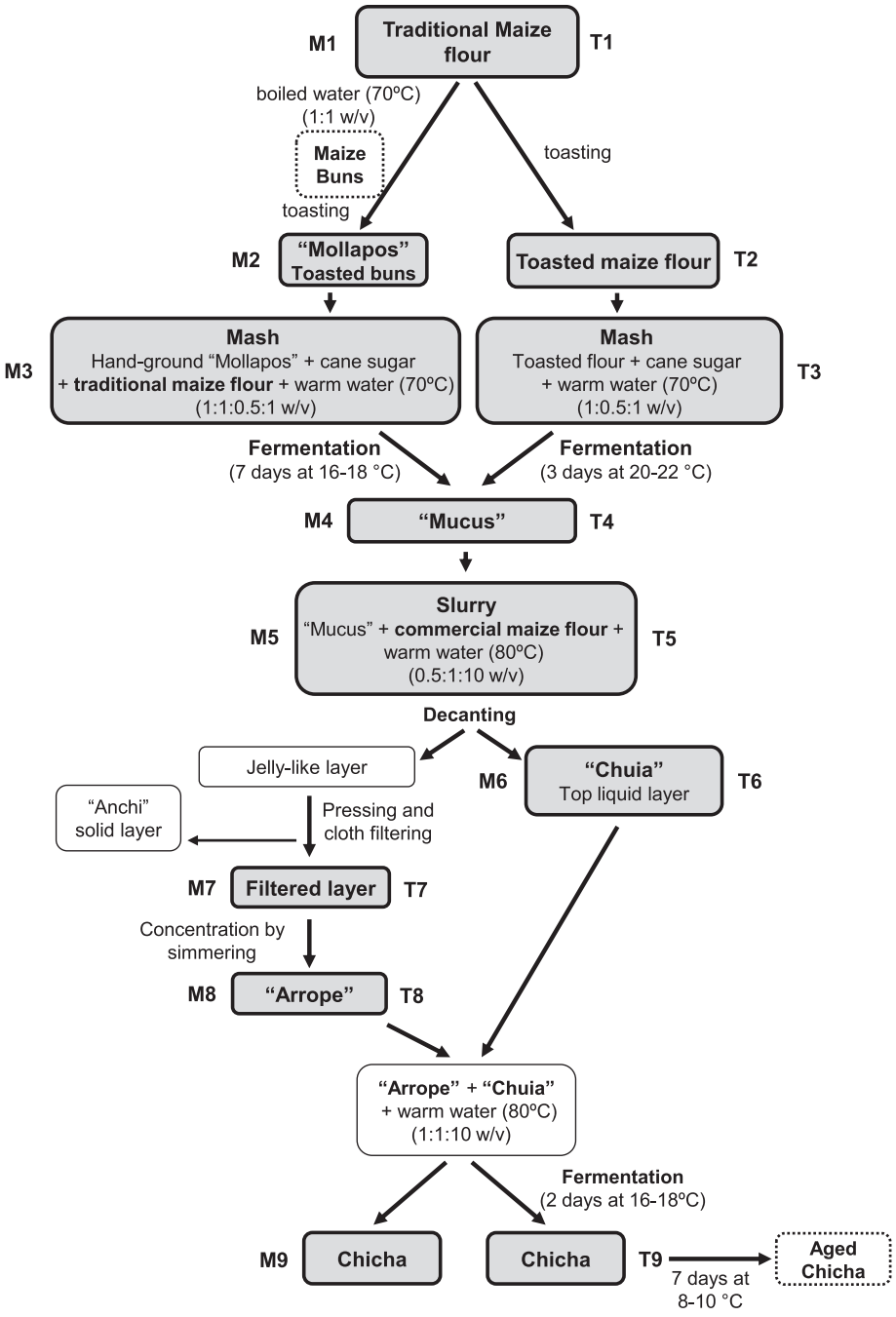

Fig. 1. Flow-chart depicting the manufacturing process of the maize-based beverage *chicha*. Samples taken from Maimará (M) and Tumbaya (T) *chichas* are indicated with M and T, respectively, followed by a number. Samples from Buns and Aged *Chicha* were also taken.

compared against NCBI (www.ncbi.nlm.gov) using the basic local alignment search tool (BLAST).

2.6. Inter-delta genotyping of Saccharomyces cerevisiae isolates

Inter-delta elements PCR amplification was carried out using delta12 (5-TCAACAATGGAATCCCAAC-3) and delta21 (5-CATCTTAACACCGTATATGA-3) primers described by Legras and Karst (2003). PCR amplification was carried out as following: 4 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 30 s at 46 °C and 90 s at 72 °C and a final step of 10 min at 72 °C. PCR products were separated in 2% agarose gels containing RedSafe (iNtRON Biotechnology Inc., Korea) at 5% dilution in 1× TAE buffer (40 mM Tris-HCl, 11.4 ml/l Acetic acid, 2 mM EDTA). Fragment lengths were estimated by comparison with a 100 bp DNA ladder (Invitrogen, USA).

2.7. Total DNA extraction and generation of D1/D2 26S rRNA gene amplicons for 454 GS Junior pyrosequencing

2.7.1. Total DNA extraction

Homogenized *chicha* samples were separated in 1 ml aliquots and used for total DNA extraction using a modification of the method proposed by Cocolin et al. (2002). In summary, frozen

samples were thawed and centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was discarded and the pellet was resuspended in sterile saline solution and centrifuged at 12,000 rpm for 10 min at 4 °C. The pellet was again resuspended in saline solution and transferred to a microcentrifuge tube containing 0.3 g of 0.5 mm diameter glass beads. The mixture was centrifuged at 12,000 rpm for 10 min at 4 °C and the supernatant discarded. The pellet was resuspended in 300 μl of lysis buffer (2% Triton 100×, 1% SDS, 100 mM NaCl, 10 mM Tris pH 8, 1 mM EDTA pH 8) and 300 μl of cold phenol-chloroform-isoamyl alcohol (25:24:1) (Sigma, USA). The cells were homogenized in a Mini BeadBeater 8 (Biospec Products, USA) three times for 30 s at maximum speed at room temperature. Tubes were maintained on ice and 300 μl of TE (10 mM Tris, 1 mM EDTA pH 7.5) were added and the mixture was centrifuged at 12,000 rpm for 10 min at 4 °C. The aqueous (upper) layer was collected and the DNA precipitated with 1 ml of −20 °C absolute ethanol. After centrifugation at 12,000 rpm for 10 min at 4 °C the pellet was dried under vacuum at room temperature and resuspended in 25 μl of sterile milli-Q water. DNA samples were quantified using a NanoDrop ND-1000 (Thermo-Fisher, USA) and stored at −20 °C. From each *chicha* sample three DNA extractions showing similar good quality and quantity were selected and pooled for PCR amplification.

2.7.2. Generation of D1/D2 26S rRNA gene amplicons for pyrosequencing

PCR amplification was carried out using fusion primers consisting of a 25-mer sequence required by 454 sequencing, followed by multiplex identifier (MID) sequences consisting of a 10-mer used to barcode each sample and, finally, the universal primers for amplification of the target sequence D1/D2 of the 26S rRNA gene (NL1 and NL4 primers described above) as required by the 454 GS Junior system (Roche, Switzerland). After PCR reaction D1/D2 26S rRNA gene amplicons from each *chicha* sample were thus identified by a different MID. PCR reactions were carried out in 25 μl final volume containing 2.5 μl of 10 × buffer, 100 μM deoxynucleotides, 0.3 μM of each primer, 1.5 mM of $MgCl_2$, 0.15 units of Taq polymerase (Takara Bio Inc., Japan) and 150–200 ng of DNA. PCR reactions were setup on ice and then directly heated to denaturation temperature in an Eppendorf Mastercycler (Eppendorf, Germany). PCR was carried out under the following conditions: initial denaturation at 94 °C for 5 min, 22 cycles of denaturation at 94 °C for 30 s, annealing at 52.5 °C for 30 s, extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min and a holding step at 4 °C. PCR amplification of each sample DNA was done in triplicate. PCR products were analyzed in 1% agarose gel containing RedSafe (iNtRON Biotechnology Inc., Korea) at 5% dilution in 1× TAE buffer. Fragment length was estimated by comparison with a 100 bp DNA ladder (Invitrogen, USA). PCR products within the expected size range were excised from the gel and purified using the High Pure PCR Purification kit (Roche, Switzerland). Purified PCR products were quantified using a NanoDrop ND-1000 (Thermo-fisher, USA). Three PCR products from each *chicha* sample showing similar band intensity and DNA concentration were pooled together. Pooled PCR products were analyzed by quality, integrity and concentration using a Bioanalyzer 2100 with high-sensitivity chip (Agilent, USA). Equimolar concentrations of PCR products from all *chicha* samples were pooled together and sequenced at the Genomic Service of the SCSIE (University of Valencia, Spain) using a 454 GS Junior (Roche, Switzerland). For sequencing the forward primer NL1was used. The datasets of raw reads are freely available from the EBI database under the project accession number PRJEB17661.

2.8. Bioinformatics analysis of D1/D2 26S rRNA gene sequences

Raw reads were filtered according to the 454 processing pipeline and separated into the different *chicha* samples according to MID barcoding. Cutadapt 1.2 (Martin, 2011) was used to remove primer sequences from the reads. Sequence processing and clustering were performed with Mothur v.1.32.1 (Schloss et al., 2009). Sequences of length <350 nt and minimum Phred score of 30 averaged over a 50 bp moving window were removed from the analysis. Chimeras detected using Uchime chimera detection program (Edgar et al., 2011) were rejected. Filtered reads were aligned, dereplicated and clustered at a sequence identity level of 98.5% using mothur (Schloss et al., 2009). Centroid sequences representative of each cluster were used for BLASTn searches against the RDP fungal database (Ribosomal Database Project, Michigan University) release 11.4. Taxonomic identity of the resulting OTUs was verified using the BLASTn tool in the GenBank nucleotide database. Diversity estimated by OTU richness, indices (Chao 1, ACE and Shannon) and rarefaction was analyzed with Mothur (Schloss et al., 2009).

2.9. Statistical analysis and graphical representation

NTSYS package version 2.21p (Exeter Software, USA) was used for constructing an UPGMA dendrogram based on a similarity matrix (Jaccard Similarity Index) generated from a table compiling presence (1) or absence (0) of same size bands in the inter-δ electrophoretic patterns (Supplementary Material Table 1). Excel 2010 (Microsoft Corp., USA) was used for species frequency analysis and abundance graphics. Vegan package for R (Oksanen et al., 2013) was used for clustering and heatmap analysis. Cultivated samples showing null colony count were excluded from the analysis. Filamentous fungi reads were also excluded from the analysis as only yeasts were recovered from the cultivated samples.

3. Results

3.1. Physicochemical analysis of chicha samples

Table 1 compiles the main physicochemical characteristics of the maize-based *chicha*. Measurements were taken from maize flour, pre-fermentative stage and final product. Evolution of pH was similar in Maimará (M) and Tumbaya (T) *chicha*. The initial pH value was above 5 and decreased approximately 2 units in the final product. Titratable acidity increased from 0.35 to 2.30 g/l of lactic acid in *chicha* M and from 0.25 to 4.30 g/l in *chicha* T. Starch content decreased from 54.52% to a final content of 2.72% in *chicha* M and from 73.11% to 2.42% in *chicha* T. In both productions, alcohol concentration at the end of the process was around 10 g/l.

3.2. Yeast counts during chicha production

Table 2 shows total yeast counts from MEA and GPYA media throughout 20 samples from M and T *chicha* fermentations. Yeast counts in maize flour showed an initial yeast population in both productions lower than 10^2 CFU/g. In *chicha* M yeast counts increased to 10^6 CFU/g at the beginning of the fermentative stage (M4), decreased in diluted samples M5 and M6 and increased again after concentration step M8. On the other hand, the highest yeast counts in *chicha* T occurred in pre-fermentative sample T3 (10^4 CFU/g) and fermentative sample M4. No yeast colonies were detected after maize flour toasting in samples M2 and T2, in M6 and

Table 1
Main physicochemical characteristics of initial, pre-fermentative and final *chicha* samples collected in Maimará (M) and Tumbaya (T) villages.

Samples	Maize Flour		Pre-fermented mash		*Chicha*	
	M1	T1	M3	T3	M9	T9
pH	5.71 ± 0.05	5.32 ± 0.13	5.07 ± 0.04	5.19 ± 0.05	3.76 ± 0.07	3.56 ± 0.09
Titratable acidity (g lactic acid/l)	0.35 ± 0.02	0.25 ± 0.04	2.20 ± 0.11	0.97 ± 0.06	2.32 ± 0.09	4.30 ± 0.12
Starch (g/100 g)	54.52 ± 2.09	73.11 ± 2.78	29.12 ± 1.90	22.57 ± 1.20	2.72 ± 0.15	2.42 ± 0.20
Ethanol (g/l)	ND	ND	1.32 ± 0.27	0.75 ± 0.31	9.45 ± 0.71	9.98 ± 0.25

Values are means of duplicate measurements ± standard deviations.
ND, not detected.

Table 2
List of species identified, colonies isolated and yeast counts from each *chicha* sample at Maimará (M) or Tumbaya (T) locations. Samples, Maize-Buns (Buns) and Aged *chicha* (Aged), do not have equivalent samples in T and M *chicha* productions respectively.

Species	M1	Buns	M2	M3	M4	M5	M6	M7	M8	M9	T1	T2	T3	T4	T5	T6	T7	T8	T9	Aged
Candida parapsilosis		29											51	1	4					
Candida zeylanoides								7	4											
Cryptococcus carnescens	1																			
Cryptococcus flavescens	9			11							1									
Cryptococcus magnus				1																
Cryptococcus nemorosus											2									
Hanseniaspora uvarum					3															
Kluyveromyces marxianus								1												
Meyerozyma guilliermondii														3						
Pichia membranifaciens				9											3		5			
Pichia sp.	16			26							21									
Rhodotorula mucilaginosa				1									5				1			
Rhodotorula slooffiae													2							
Saccharomyces cerevisiae		10		6	87	25		57	84	38				19	2		24			7
Torulaspora delbrueckii																	1			
Wickerhamomyces anomalus				2										17						
Total	**26**	**39**	**0**	**56**	**90**	**25**	**0**	**65**	**88**	**38**	**24**	**0**	**58**	**40**	**9**	**0**	**31**	**0**	**0**	**7**
CFU/g	**$\leq 10^2$**	**1.5×10^3**	**0**	**3.8×10^3**	**5×10^6**	**3.5×10^3**	**0**	**1.2×10^2**	**2.6×10^6**	**4.8×10^2**	**$\leq 10^2$**	**0**	**4.7×10^4**	**1.2×10^3**	**≤ 10**	**0**	**$\leq 10^2$**	**0**	**0**	**≤ 10**

T6 after decanting of slurry or in T8 and T9 after concentration by simmering of filtered layer T7.

3.3. Yeast identification and cultivable species diversity during chicha production

Five hundred and ninety-six yeast colonies were isolated from Maimará and Tumbaya *chicha* productions. A tentative identification of yeast isolates by PCR-RFLPs of the 5.8S-ITS rDNA region revealed the presence of 16 different yeast species confirmed by sequencing of the D1/D2 domains of 26S rDNA gene (Table 2). *S. cerevisiae* dominated the fermentative steps of both *chicha* productions. Isolates represented around 72% and 31%, respectively, of the total yeast population. The second and third most abundant isolated yeast species in the pre-fermentative steps, 1 to 3, were *Candida parapsilosis* and *Pichia* sp. NRRL Y-17803. A moderate number of *Cryptococcus* isolates (*chicha* M) and *Rhodotorula* isolates (*chicha* T) were also found.

3.4. Amylase activity of yeast isolates

Amylolytic activity was qualitatively determined for all isolates of the 16 identified yeast species (Table 3). The plate method revealed that *Cryptococcus* isolates showed the highest enzymatic activity.

Table 3
Percentage of *chicha* isolates showing amylase activity.

Species	Amylolytic activity[a]			
	-	w	+	++
Candida parapsilosis	28	53	19	0
Candida zeylanoides	88	12	0	0
Cryptococcus carnescens	0	0	0	100
Cryptococcus flavescens	0	0	0	100
Cryptococcus magnus	0	0	0	100
Cryptococcus nemorosus	0	0	0	100
Hanseniaspora uvarum	100	0	0	0
Kluyveromyces marxianus	100	0	0	0
Meyerozyma guilliermondii	100	0	0	0
Pichia membranifaciens	47	21	32	0
Pichia sp.	81	11	8	0
Rhodotorula mucilaginosa	0	0	100	0
Rhodotorula slooffiae	0	100	0	0
Saccharomyces cerevisiae	72	21	7	0
Torulaspora delbrueckii	100	0	0	0
Wickerhamomyces anomalus	0	54	46	0

[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).

3.5. Molecular typing of S. cerevisiae isolates

Genetic diversity of isolates within *S. cerevisiae* was evaluated by PCR amplification of inter-δ elements (delta elements). Electrophoretic patterns of delta elements consisted of 5–12 bands between 100 bp to 2000 bp. Up to 42 different inter-δ patterns were found in two hundred and thirteen isolates of *S. cerevisiae* from *chicha* M whereas only one pattern (type 25) was shared by 52 *S. cerevisiae* isolates from *chicha* T (Fig. 2). The similarity matrix (Supplementary Material Table 2) indicated a high level of heterogeneity between patterns as more than 70% of the similarity values were below 0.5 similarity. This is also evident in the UPGMA clustering where few groups of patterns were observed at high similarity values (Fig. 2). Nevertheless, despite the large differences between patterns, five bands indicated with black arrows were present in most patterns. Distribution of patterns by samples (Supplementary Material Table 3) showed that patterns 2, 8a, 12a and 21 were the most abundant, displayed by more than 25 strains each and present in most *chicha* M samples. On the other hand, 21 patterns were displayed by single isolates. Our results also indicated that the highest number of different patterns was observed in fermentative steps M4 to M8.

3.6. Diversity during chicha production determined by pyrosequencing

A total of 242,993 raw reads were obtained by pyrosequencing and, from these, 214,291 reads passed the quality filters applied (Table 4). The lowest and highest number of high quality reads per sample were 6189 (T1) and 15,998 (M4). Diversity analysis at 97%

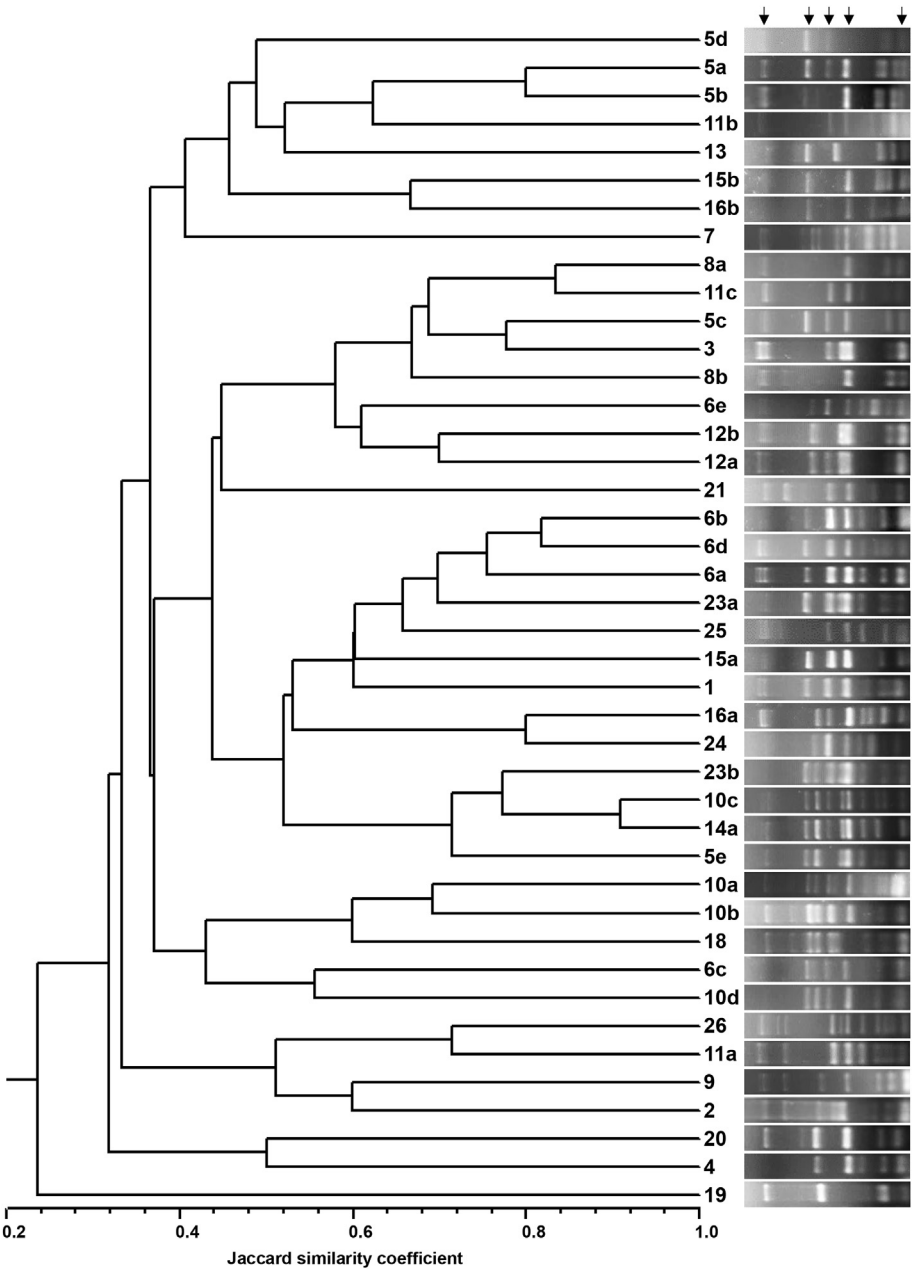

Fig. 2. UPGMA dendrogram analysis showing the relationships among *S. cerevisiae* strains. Each inter-delta pattern is labelled with a number. Bands found shared by several patterns are indicated with an arrow.

Table 4
Summary of 454 pyrosequencing data, OTU richness and diversity indices (Shannon, Chao1 and ACE) for fungal D1/D2 26S rDNA libraries from *chicha* samples.

	Maimará *chicha*									Tumbaya *chicha*								
	M1	Buns	M2	M3	M4[a]	M5[a]	M6	M7	M8	M9[a]	T1	T2	T3	T4	T5	T6	T7	T8	T9	Aged
RR	11582	12189	11169	11240	16801	16092	11705	14105	11609	15963	7589	8200	14804	12396	9963	10433	10980	13703	10523	11947
HQR	9986	9958	10143	9831	15998	15176	10634	12383	10148	14745	6819	7390	13522	9348	9023	8841	9680	12403	9087	9177
OTU	69	75	64	76	26	18	61	79	19	20	81	63	33	39	9	19	11	67	29	20
Shan	1.75	2.19	1.20	2.14	0.12	0.13	1.20	1.60	0.06	0.15	1.79	1.29	1.40	0.69	0.04	0.26	0.05	1.14	0.92	0.29
Chao	88.09	103.88	83.09	99.33	31	24	95.20	122.15	31	35	151.13	80.10	54	43.67	15	33	12	79.67	120	29
ACE	107.98	98.74	102.43	97.46	32.33	19.98	78.50	185.33	27.38	35.84	198.32	81.52	109.55	46.13	21.49	37.70	11.93	88.33	92.09	35.99

RR, raw reads; HQR, high quality reads; OTU, number of OTUs at 3% divergence level; Shan, Shannon diversity index; Chao, Chao1 richness index; ACE, ACE richness index.
[a] Samples M4, M5 and M9 are the result of merging two technical duplicates (454 Junior sequencing duplicates).

sequence similarity produced between 9 (T5) and 81 (T1) OTUs per sample. Diversity indexes were higher in pre-fermented than in fermented samples, with the exception of M6, M7 and T8. Indeed, rarefaction curves at 3% (Supplementary Material Fig. 1) revealed that in some pre-fermentative samples saturation was not reached at the achieved sequencing depth. On the other hand, rarefaction curves of fermentative samples flattened over time.

BlastN analysis of high quality reads against the RDP database fungi 11.4 allowed classification of D1/D2 26S rDNA gene sequences into filamentous fungi and yeast taxa (Supplementary Material Table 4). A detailed exploration of the BlastN results revealed the presence of (ca.) 10000 reads that matched to database entries that could be identified as chimera. All reads corresponding to these chimeras were also eliminated from the high quality reads (Supplementary Material Table 5). Pyrosequencing of D1/D2 regions of 26S rDNA gene allowed detection of more than one hundred yeast species and almost fifty filamentous fungi taxa. Table 5 shows filamentous fungi genera and yeast species with abundances of more than 1% of the total reads. Yeasts were always more abundant than filamentous fungi except in initial samples M1 and T1 (Supplementary Material Fig. 2; Supplementary Material Tables 4 and 6). Among the filamentous fungi, the genus *Fusarium* was the most abundant. In case of yeasts, the most abundant species was *S. cerevisiae* representing more than 90% of the reads per sample in most fermentative samples. Following in abundance were species of genera *Kluyveromyces*, *Pichia*, *Debaryomyces* and *Candida* which were the dominant yeasts in pre-fermentative steps 1 to 3 and abundant in fermentative samples when *S. cerevisiae* was below 90%. *Kluyveromyces marxianus* dominated in samples M2 and T2 as well as in T8. A novel species of the genus *Pichia* similar to the strain NRRL Y-17803 in its D1/D2 26S rDNA gene sequence was detected in pre-fermentative samples of both *chicha* productions and represented more than 1% of reads in Maimará *chicha* samples M1 and M3. Species of other yeast genera appeared in different samples along the fermentative process (Table 5).

Clustering of *chicha* samples in a heatmap summarizes the differences between *chicha* M and T in terms of abundance of yeasts taking into account species isolated as well as those represented by more than 1% of the reads by pyrosequencing (Fig. 3). The heatmap revealed a clear separation of fermentative samples (A) from pre-fermentative samples (B, C and D). In cluster A (fermentative), sequenced samples and their cultivated counterparts from both *chicha* fermentations appear intermixed. However, pre-fermentative samples appear to be distributed in three clusters. A clear separation between sequenced (cluster B) and cultivated (cluster C) samples could be observed. Cluster D comprised sequenced and cultivated samples; although both types of samples appeared separated in sub-clusters. The clustering analysis also showed that microbiota profiles of pre-fermentative samples from Maimará and Tumbaya *chicha* did not cluster together directly. The only exceptions were cultivated samples M1-C and T1-C, which grouped together in cluster D, as well as sequenced samples M2-S and T2-S that clustered together in cluster B. Fig. 3 also exposed discrepancies between cultivated and sequenced samples. Species *Candida oleophila*, *Debaryomyces hansenii*, *Galactomyces candidum*, *K. lactis*, *M. caribbica*, *P. fermentans* and *Trichosporon domesticum*, among the most abundant species found by pyrosequencing, were not recovered by cultivation. On the contrary, cultivated species such as *C. parapsilosis*, *Cryp. carnescens*, *Cryp. flavescens*, *Cryp. nemorosus*, *R. slooffiae* and *Torulaspora delbrueckii*, represented less than 1% of the reads in all sequenced samples. Moreover, very few reads were found for cultivated species in most Tumbaya *chicha* samples (Table 4 Supplementary Material). The second most abundant species detected by pyrosequencing, *K. marxianus*, was only found in one colony isolated from sample M7.

Table 5
Relative abundances of filamentous fungi genera and yeast species based on taxonomic assignations of D1/D2 26S rDNA reads from *chicha* fermentation samples. Only taxa accounting for reads above 1% are shown.

	M1	Buns	M2	M3	M4	M5	M6	M7	M8	M9	T1	T2	T3	T4	T5	T6	T7	T8	T9	Aged
Acremonium	10.40			12.49							11.10									
Cladosporium											3.64									
Fusarium	56.38	26.39	2.33	33.83							60.24									
Mucor	6.00	4.16									2.02									
Penicillium																5.10				
Peyronellaea											1.09									
Candida oleophila	11.31	18.79	1.68	8.75							1.29	3.46								
Candida zeylanoides														7.23						
Cryptococcus magnus														2.88						
Debaryomyces hansenii		3.38	12.44	2.74			4.99	3.67			1.88	13.86	20.26					19.81	3.79	
Galactomyces candidum											1.95									
Hanseniaspora uvarum								5.60			1.66									
Kluyveromyces lactis		2.91	9.13	17.24			9.54				2.73	12.31						5.46		
Kluyveromyces marxianus	3.18	12.79	63.48	2.83			6.55	15.85			7.58	59.23						62.69	14.66	4.81
Meyerozyma caribbica	1.68	3.69	1.10	2.51																
Meyerozyma guilliermondii		1.20	1.02									1.79						2.91		
Pichia fermentans							1.71	27.97				4.42						1.77		
Pichia sp. NRRL Y-17803	6.77	17.26	2.95	10.85									36.56							
Rhodotorula mucilaginosa							4.54													
Saccharomyces cerevisiae				1.02	98.72	99.78	68.42	42.55	99.61	99.68			9.33	86.77	99.72	94.76	99.58	1.78	78.96	93.56
Trichosporon domesticum			1.20									1.50						1.81		
Wickerhamomyces anomalus													32.18							

4. Discussion

Chicha manufacturing is a fermentative process in which starch is hydrolyzed into sugars used for ethanol production. *Chicha* can be prepared from various cereals although the most common in the Andean region is maize (Faria-Oliveira et al., 2015). Physicochemical characterization of Maimará and Tumbaya maize-based *chicha* revealed values of pH and ethanol comparable to those found in other maize-based beverages such as *champús* (Osorio-Cadavid et al., 2008) and *ogi* (Omemu et al., 2007). Cultivable yeast population declined to zero in some fermentation steps likely due to high baking temperatures in pre-fermentative step 2 and high simmering temperature in step T8, as well as after *chicha* dilution in steps 6 and 9. In a similar study, Elizaquivel et al. (2015) reported absence of microbial growth from samples of maize flour baking in a clay oven for 1.5 h. Despite the significant impact of high temperature and dilution on yeast counts, the highest CFU/g values found in our study were similar to those found in maize-based beverage *mawè* (Greppiet al., 2013), but lower than those found in *champús* (Osorio-Cadavid et al., 2008).

The increase in ethanol concentration along *chicha* fermentation prevented growth of most yeast species except for *S. cerevisiae*, as occurs in other alcoholic beverages (Fleet, 2006). The increase in alcohol content was concomitant with the increase in *S. cerevisiae* counts. The largest increase in *S. cerevisiae* counts was observed after sugar addition and fermentation of mash (step 4). Addition of sugar and warm water is used in most maize based food and beverages for increase of the alcohol content (Lorence-Quiñones et al., 1999). Notwithstanding the clear dominance of *S. cerevisiae* in both *chicha* fermentations, the significantly larger number of isolates found in Maimará in comparison with Tumbaya *chicha* indicated differences between both fermentations. *S. cerevisiae* isolates from Maimará *chicha* displayed a rich genetic heterogeneity whereas only one inter-δpattern was detected in *S. cerevisiae* isolates from *chicha* T. A similar diversity richness of inter-δpatterns in *S. cerevisiae* isolates, as found in *chicha* M, has been reported along the spontaneous fermentation of wine in Argentina (Mercado et al., 2007). On the other hand, detection of only one inter-δpattern in *chicha* T would be in agreement with prevalence in pots and surfaces routinely used for *chicha* production of an adapted microbiota as reported in previous studies (Steinkraus, 1996; Faria-Oliveira et al., 2015).

Pyrosequencing revealed a more complex picture of *chicha* fermentation and a large number of filamentous fungi and non-Saccharomyces yeast species not detected by cultivation appeared to dominate the early steps of the fermentative process. These findings are in agreement with previous studies in other food and beverages (Bokulich et al., 2012; Illeghems et al., 2012; David et al., 2014).

Diversity estimators indicated that samples along the *chicha* production were very unequal. Pre-fermentative samples were characterized by many species represented by very few reads, whereas most fermentative samples were distinguished by few species represented by many reads. This reduction in diversity displayed well the yeast selection process occurring during the fermentation.

Pyrosequencing results revealed a large number of yeast species not recovered by cultivation. The cultivation-based method detected very few yeast taxa, most of them corresponding with very few or zero reads in the pyrosequencing analysis. Insufficient sequencing depth would prevent detection of yeast species isolated by cultivation. Rarefaction curves indicated that diversity was well represented in fermentative samples whereas in pre-fermentative samples diversity seemed to be under-represented, as observed in similar studies on wine or sausages fermentation (David et al.,

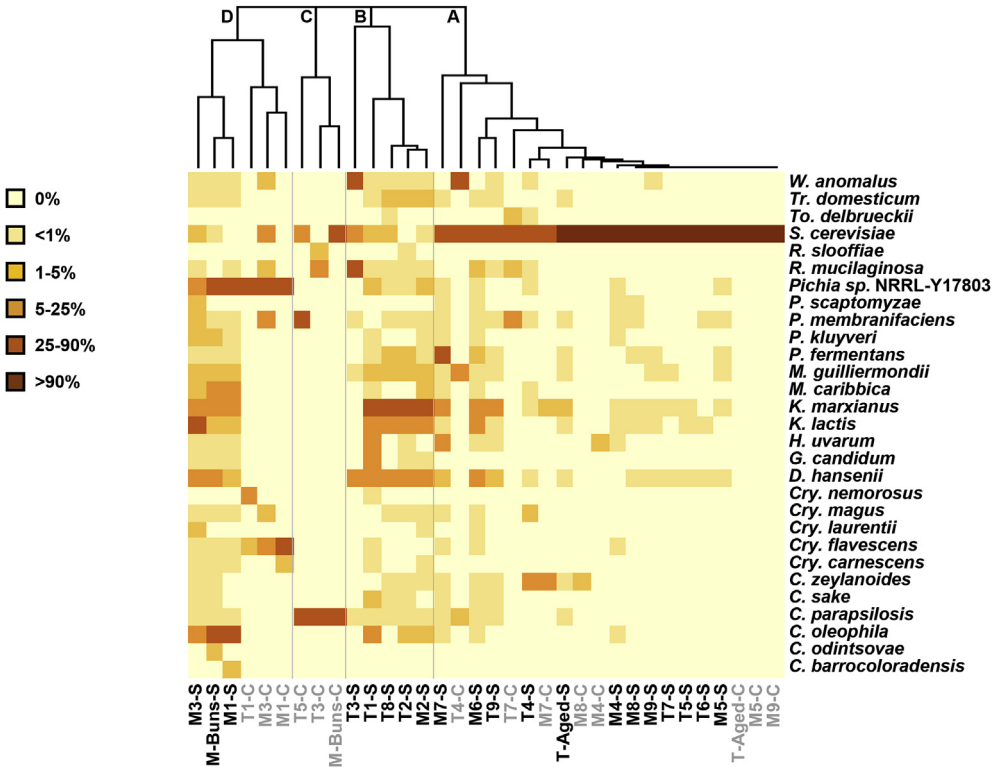

Fig. 3. Heatmap showing yeast species abundance and distribution per sample of *chicha* M and T. Species detected in samples via cultivation C (light grey) or sequencing S (black) are included. Species accounting for reads above 1% of isolates or reads are displayed.

2014; Greppi et al., 2015). However, the large number of reads per sample allowed detection of yeast species represented by a very low number of reads, therefore the sequencing depth should be able to detect the cultivated yeast species in acceptable numbers (David et al., 2014; Stoops et al., 2015; Quigley et al., 2012; Lusk et al., 2012; Greppi et al., 2015).

On the other hand, it is well known that cultivation is strongly influenced by the physiological state of the cell, as well as the composition of the culture media used or any enrichment step applied before plate count (Kurtzman et al., 2011). The four non-selective culture media used in our study favored isolation of species represented by a very low number of reads in the pyrosequencing results. Among these, *C. parapsilosis*, *R. slooffiae* and several species in the genus *Cryptococcus* have not been reported associated with fermented cereal-based products (Gotcheva et al., 2000; Omemu et al., 2007; Lacerda Ramos et al., 2010; Greppi et al., 2013). These studies as well as our data would indicate that isolation of these species possibly be cultivation artifacts due to manufacturing practices during the *chicha* production. On the other hand, isolation of non-*Saccharomyces* yeasts, dominant in pre-fermentative steps and detected by pyrosequencing, was not possible using our cultivation approach. Nutritional requirements of yeast species *C. oleophila*, *D. hansenii*, *K. lactis* and *K. marxianus* were fulfilled by the culture media used for isolation (Kurtzman et al., 2011), however our results indicate that additional enrichment steps or media for selective isolation for these species should be included in future explorations of yeast diversity.

The cultivation-based approach made possible the isolation of a large number of isolates of a new *Pichia* species. Results from cultivation and pyrosequencing supported that this new *Pichia* species (*Pichia* sp. NRRL Y-17803) seemed to be associated to fermentation steps where traditional or *criollo* maize flour was added to the fermentative process. Presently, further studies are being done to accommodate these isolates into a new *Pichia* species.

In conclusion, yeast diversity of *chicha* beverage was evaluated by the combined use of the NGS and culture-dependent methods. Both methods identified *S. cerevisiae* as the dominant yeast during *chicha* production; therefore, pyrosequencing seems to be of limited added value for the study of environments dominated by a low number of species. On the other hand, pyrosequencing allowed detection of non-*Saccharomyces* yeast species dominating pre-fermentative steps. These non-*Saccharomyces* yeast species might have a secondary role, different from alcohol production, in *chicha* fermentation like occurs in well-known alcoholic beverages (Jolly et al., 2006).

To our knowledge this is the first study about diversity of yeast species during the manufacturing process of the maize-beverage *chicha*. Further studies on *S. cerevisiae* and non-*Saccharomyces* yeasts present in *chicha* are required to understand their role on the production of this beverage, their contribution to *chicha* nutritional value and microbial safety as well as to sensory quality before commercialization of this beverage.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2017.05.007.

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