Yeast diversity during the fermentation of Andean *chicha*: a comparison between 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. Classical microbiological methods allowed isolation of five hundred ninety-six yeasts identified into 16 species 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 allowed detection of 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 examination 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, different from *S. cerevisiae*, dominated the pre-fermentative steps and abounded in fermentative 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 none or very few reads in the pyrosequencing analysis.

Keywords: *chicha*; yeast; diversity; cultivation; Next Generation Sequencing; *S. cerevisiae* typing

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 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, being maize the most representative and consumed among cereals (Perry et al., 2006). Moreover, Andean cereals and particularly maize are the substrate 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 Barragan, 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., 1993). 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 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 populations dynamics have been poorly studied and their role during the elaboration 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 have 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. Figure 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 mixing the toasted 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 room temperature. The pre-fermented mass (*mucus*) was then supplemented with commercial maize flour and warm water obtaining a

slurry which was thoroughly mixed and allowed to cool and decant for 2-3 hours. The top liquid layer (*chui*a) was separated from the jelly-like middle layer which was pressed, filtered and concentrated by simmering. This concentrated and caramelized layer (*arrope*) was then mixed with the top layer (*chui*a) and diluted with water to constitute the Maimara *chicha*. In case of Tumbaya *chicha* the mix of *arrope* and *chui*a was allowed to ferment for 2 additional days. Tumbaya *chicha* was then stored at low temperature for 7 to 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 analyzed on 10 ml of each sample using NaOH 0.1 N and phenolphthalein as indicator. Starch content was determined using the method described by Tovar et al. (1990). Ethanol concentration was determined using an enzymatic method (R-Biopharm AG-Germany).

2.3 Yeast enumeration and isolation

For yeasts enumeration and isolation during *chicha* production, 5 g of each sample was resuspended in 45 ml of sterile saline solution (0.9%) and homogenized for 3 times at 30 s using a Pulsifier (Microgen Bioproducts, 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 to 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 the ITS1-5.8S-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 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 1x 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 100X, 1% SDS, 100mM NaCl, 10mM Tris pH 8, 1mM EDTA pH 8) and 300µl 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 (10mM Tris, 1mM 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 identifiers (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 x buffer, 100 µM deoxynucleotides, 0.3 µM of each primer, 1.5mM of MgCl₂, 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 1x 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 NL1 was 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 (Table 1 Supplementary Material). 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 inferior to 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 raised 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 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). *Saccharomyces cerevisiae* was the dominant species in the fermentative steps of both *chicha* productions. Isolates from this species 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. Together with these species a moderate number of *Cryptococcus* isolates in *chicha* M and *Rhodotorula* isolates in *chicha* T were 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 all species belonging to *Cryptococcus* genus showed the highest enzymatic activity. Strains of *Rhodotorula mucilaginosa* and *R. slooffiae* showed also high levels of amylolytic activity. On the contrary, strains pertaining to ascomycetous species displayed negative or weak amylase activity included *S. cerevisiae*.

3.5 Molecular typing of *S. cerevisiae* isolates

Genetic diversity within *S. cerevisiae* strains was evaluated by inter- δ PCR amplification (delta elements). Inter- δ patterns were defined by 5 to 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 was shared by all *S. cerevisiae* isolates (52) from *chicha* T (Figure 2). The similarity matrix (Table 2 Supplementary Material) indicated a high level of heterogeneity between patterns as more than 70% of similarity values were below 0.5 similarity. This is also evident in the UPGMA clustering where few

groups of patterns are observed at high similarity values. Nevertheless, despite the large differences between patterns, five bands indicated with black arrows were present in most patterns. Distribution of patterns by samples (Table 3 Supplementary Material) 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 filters applied (Table 4). The lowest and highest number of high quality reads per sample were 6,189 (T1) and 15,998 (M4). Diversity analysis at 97% sequence similarity produced between 9 (T5) and 81 (T1) OTUs per sample. Diversity indexes were higher in pre-fermentative than in fermentative samples, with the exception of M6, M7 and T8. Indeed, rarefaction curves at 3% (Figure 1 Supplementary Material) 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.

Blasting of high quality reads against the RDP database allowed classification of D1/D2 26S rDNA gene sequences into filamentous fungi and yeast taxa (Figure 3; Table 4 Supplementary Material). A detailed exploration of the BlastN results revealed further chimeric sequences which were also eliminated from the high quality reads (Table 5 Supplementary Material). In case of filamentous fungi, identification was achieved at genus level or above, whereas in case of

yeasts, identification was at species level. Pyrosequencing of D1/D2 of 26S rDNA gene allowed detection of more than one hundred yeast species and almost fifty filamentous fungi taxa. Figure 3 showed that the total number of yeasts in *chicha* samples was always higher than the number of filamentous fungi except in initial samples M1 and T1. Moreover, in the majority of fermentative samples of both *chicha* productions, as well as in pre-fermentative Tumbaya samples, the number of filamentous fungi reads represented less than 1% of the reads per sample. Nevertheless, a slight increase in the total number of filamentous fungi reads could be observed after addition of maize flour to the fermentative process in samples M3, M6 as well as T6.

The detailed analysis of the identification results revealed that very few taxa were represented by more than 1% of reads per sample (Figure 3; Figure 2 Supplementary Material; Table 4 Supplementary Material; Table 6 Supplementary Material). Among the filamentous fungi, the genus *Fusarium* was the most abundant followed by *Acremonium* and *Mucor*. *Penicillium* could be found above 1% reads in sample T6. In the 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 in percentages below 90%. The species *Kluyveromyces marxianus* was the most abundant after *S. cerevisiae* and dominated in samples M2 and T2 as well as 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

369 and represented more than 1% of reads in Maimará *chicha* samples M1 and
370 M3. *Pichia fermentans* was the second most abundant species in sample M7.
371 The most abundant *Candida* species was *C. oleophila*, which appeared only in
372 pre-fermentative samples of both *chicha* productions. *Meyerozyma caribbica*
373 was detected only in pre-fermentative samples in Maimará *chicha* and
374 *Wickerhamomyces anomalus* appeared only in sample T3. Non-fermentative
375 yeast species were found in low number in both *chicha* fermentations, although
376 *Rhodotorula mucilaginosa* was the most abundant species in sample T3.
377 Clustering of *chicha* samples in a heatmap summarizes the differences between
378 *chicha* M and T in terms of abundance of yeasts taking into account all species
379 isolated as well as those represented by more than 1% of the reads by
380 pyrosequencing (Figure 4). The heatmap revealed a clear separation of
381 fermentative samples (A) from pre-fermentative samples (B, C and D).
382 Fermentative samples were characterized by dominance of *S. cerevisiae*
383 respect to any other yeast species. In pre-fermentative samples, co-dominance
384 of several yeast species was observed in all samples. Two fermentative
385 samples, T5-C (cultivated) in cluster C and T8-S (sequenced) in cluster B were
386 the only exceptions. In cluster A (fermentative), sequenced samples and their
387 cultivated counterparts from both *chicha* fermentations Maimará and Tumbaya
388 appear intermixed. On the contrary, pre-fermentative samples appear
389 distributed in three clusters. A clear separation between sequenced (cluster B)
390 and cultivated (cluster C) samples could be observed. Cluster D comprised
391 sequenced and cultivated samples; although both types of samples appeared
392 separated in sub-clusters. The clustering analysis also showed that microbiota
393 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. Figure 4 also exposed discrepancies between cultivated and sequenced samples. Species *C. oleophila*, *Debaryomyces hansenii*, *Galactomyces candidum*, *Kluyveromyces lactis*, *M. caribbica*, *P. fermentans* and *Trichosporon domesticum*, among the most abundant species found by pyrosequencing, were not recovered by cultivation. On the contrary, species found in cultivated samples such as *C. parapsilosis*, *Cryptococcus carnescens*, *Cryp. flavescens*, *Cryp. nemorosus*, *R. slooffiae* and *Torulaspora delbrueckii*, represented less than 1% of the reads in all sequenced samples. Moreover, zero 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.

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 Argentinian 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). Declining pH values along the fermentative process did not seem to prevent yeast growth. However, 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 hours. Moreover, these authors reported isolation of bad quality amplifiable DNA for pyrosequencing from those samples, as well as from diluted *chicha* samples. On the contrary, our pyrosequencing results demonstrate that we did not find any difficulty for DNA extraction or amplification from heat damaged or diluted samples.

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 the *chicha* fermentative process challenged growth of most yeast species but for *S. cerevisiae*, as occurs in most alcoholic beverages (Fleet et al., 2006).

A direct role of the yeast isolates in starch hydrolysis for fermentable sugar accessibility and subsequent alcohol production could not be explained, as only species in the non-fermenting genus *Cryptococcus* displayed a strong amylolytic activity. Addition of warm water during *chicha* fermentation, as reported in steps 3 and 5, has been described for extracting fermentable sugars from maize in *chicha* production (Lorence-Quiñones et al., 1999). Moreover, these authors also reported addition of sugar together with warm water to increase the alcohol content of *chicha* as reported in pre-fermentative step 3. Accordingly, an increase in yeasts counts, mainly *S. cerevisiae*, could be observed from sample 3 to 4 after sugar addition. Pyrosequencing yielded a most complex picture of *chicha* fermentation, as in the same samples a change

of dominance from filamentous fungi and non-*Saccharomyces* yeasts to *S. cerevisiae* could be observed. Analysis of subsequent samples showed dominance of *S. cerevisiae* except in T8 obtained after concentration of *chicha* by simmering. It is worth noting that equivalent sample M8 showed an increase in yeast counts and in the percentage of *S. cerevisiae* reads. In summary, the culture-based approach as well as pyrosequencing confirmed previous findings regarding the dominance of *S. cerevisiae* in *chicha* fermentation (Faria-Oliveira et al., 2015).

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. Analysis of *S. cerevisiae* inter- δ patterns of isolates from both fermentation processes revealed large differences between *chicha* M and T. *S. cerevisiae* isolates from Maimará *chicha* displayed a rich genetic heterogeneity whereas only one inter- δ pattern was detected in *S. cerevisiae* isolates from *chicha* T. A similar diversity richness of inter- δ patterns 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- δ pattern 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, 1999; Faria-Oliveira et al., 2015).

Other yeast species, different from *S. cerevisiae*, were present in large numbers in most steps of *chicha* production. Pyrosequencing allowed detection of a large number of taxa, and a detailed exploration of yeast diversity in *chicha*, as already reported for bacteria or other foods (Elizaquivel et al., 2015; Bokulich et

al., 2012; Ercolini et al., 2012; van Hijum et al., 2012). Diversity estimators indicated that *chicha* samples 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. However, comparison between pyrosequencing reads percentages and number of isolates of non-*Saccharomyces* species revealed broad differences between methodologies. 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 none or very few reads in the pyrosequencing analysis.

The lack of correspondence between pyrosequencing and cultivation could be attributable to several causes. Insufficient sequencing depth in the pyrosequencing approach 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., 2014; Greppi et al., 2015). However, the large number of reads per sample, compared with similar studies, does not support this idea (David et al., 2014; Stoops et al., 2015; Quigley et al., 2012; Lusk et al., 2012; Greppi et al., 2015). Two more points to consider would be that DNA extraction and/or PCR amplification could have been less efficient in case of some non-*Saccharomyces* species (e.g. due to cell wall differences or mismatches in primer sites), however neither literature nor our pyrosequencing

results support this idea. 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). Our cultivation approach included the use of four non-selective culture media for cultivation of yeasts, however some species are represented by a higher number of isolates than expected by 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). Presence of *C. parapsilosis* in *chicha* could be attributed to manufacturing practices such as hand manipulation of maize flour by the local producers. Similarly, Elizaquivel et al. (2015) reported an increase in coliform bacteria in an equivalent step of *chicha* production. These reports as well as our data would indicate that isolation of *C. parapsilosis* and other species are cultivation artifacts without real relevance in *chicha* fermentation. On the contrary, isolation of non-*Saccharomyces* yeasts dominant in pre-fermentative steps, as shown 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 although pyrosequencing allowed detection of a large number of yeast species dominating pre-fermentative steps. A high diversity of *S. cerevisiae* strains was found by delta elements analysis, suggesting that selection of specific strains with special physiological characteristics would be necessary for starter production in commercial manufacturing of *chicha*. Few yeast isolates were able to hydrolyze starch and, moreover, the low number of reads obtained by pyrosequencing for these species indicates that this enzymatic activity does not play an important part in *chicha* production. The large number of non-*Saccharomyces* yeasts detected by pyrosequencing indicates that these species might have a secondary role, different from alcohol production, in *chicha* fermentation like occurring 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*. Artisanal Maimará and Tumbaya *chicha* fermentation processes appeared to be very similar but for few exceptions regarding some pre-fermentative steps. Application of state-of-the-art sequencing technologies to the *chicha* ecosystem revealed the extent of microbial complexity along the fermentative process, showing concomitance of different types of microorganisms such as lactic acid bacterial and other

bacteria (Elizaquivel et al., 2015) and yeasts and molds, in this study. Previous studies have confirmed the use of high-throughput sequencing technologies for exploration of food fermentations (van Hijum et al., 2012; Ercolini, 2013; Tamang et al., 2016); however, the use of a cultivation approach for profiling of microbial communities from traditional foods and beverages has a potential role as source of new microorganisms with important technological or functional properties (Bourdichon et al., 2012). Yeasts have been described as responsible for a range of functions such as fermentation of carbohydrates for production of ethanol and aromatic compounds, stimulation of lactic acid bacteria growth providing essential metabolites, growth inhibition of mycotoxigenic moulds, as well as producers of probiotics (Jespersen, 2003, Hjortmo et al., 2005; Shetty et al., 2007; Pedersen et al., 2012). 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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Figure legends

Figure 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.

Figure 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.

Figure 3. Relative abundances of filamentous fungi genera and yeast species based on taxonomic assignments of D1/D2 26S rDNA reads from *chicha* fermentation samples. Only taxa accounting for reads above 1% are displayed.

Figure 4. 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.

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		<i>Chicha</i>	
	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
Titrateable 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
<i>Candida parapsilosis</i>		29											51	1	4					
<i>Candida zeylanoides</i>								7	4											
<i>Cryptococcus carnescens</i>	1																			
<i>Cryptococcus flavescens</i>	9			11							1									
<i>Cryptococcus magnus</i>				1																
<i>Cryptococcus nemorosus</i>											2									
<i>Hanseniaspora uvarum</i>					3															
<i>Kluyveromyces marxianus</i>								1												
<i>Meyerozyma guilliermondii</i>														3						
<i>Pichia membranifaciens</i>				9											3		5			
<i>Pichia</i> sp.	16			26							21									
<i>Rhodotorula mucilaginosa</i>				1									5				1			
<i>Rhodotorula slooffiae</i>													2							
<i>Saccharomyces cerevisiae</i>		10		6	87	25		57	84	38				19	2		24			7
<i>Torulaspora delbrueckii</i>																	1			
<i>Wickerhamomyces anomalus</i>				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

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

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

* 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)

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 <i>chicha</i>										Tumbaya <i>chicha</i>									
	M1	Buns	M2	M3	M4*	M5*	M6	M7	M8	M9*	T1	T2	T3	T4	T5	T6	T7	T8	T9	Stored
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

*Samples M4, M5 and M9 are the result of merging two technical duplicates (454 Junior sequencing duplicates).

Figure 1 Mendoza et al. 2016

Figure 2 Mendoza et al. 2016

Figure 3 Mendoza et al. 2016

Figure 4 Mendoza et al. 2016

"Data matrix to test UPGMA *S.cerevisiae* Chicha"

"26 characters y 42 OTUs"

"labeled columns, no missing values"

	1	26	42	0													
	5d	5a	5b	11b	8a	6b	23a	6d	25	16a	1	5c	15a	6a	10a	10b	18
a	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0
b	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	0	0
c	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
d	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
e	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
f	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
g	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
h	1	1	1	0	0	1	1	1	0	0	1	1	1	1	1	1	1
i	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
j	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	1	1
k	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
l	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1
m	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1
n	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
o	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
p	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
q	0	0	0	0	0	1	1	1	1	1	0	0	0	1	0	0	0
r	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1
s	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0
t	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
u	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0
v	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	0	1
w	1	0	0	0	1	0	1	1	0	0	0	1	0	0	0	0	0
x	0	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0
y	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	0
z	1	1	1	1	0	1	0	1	1	1	0	1	1	1	1	1	1

[illegible]

8b	9	2	16b	20	4	24
0	0	0	0	0	0	0
1	1	0	0	0	0	1
1	1	1	0	1	0	1
0	0	0	0	0	0	0
1	1	1	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	1	0	0
0	0	0	1	0	0	0
0	0	0	0	0	0	0
0	0	0	0	1	1	1
0	1	1	0	0	0	0
0	0	0	0	0	0	1
0	0	1	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
1	1	1	1	1	1	1
0	0	0	0	0	1	1
0	0	0	0	0	0	0
0	0	0	1	0	0	1
0	0	1	0	0	0	0
0	1	0	1	0	0	0
0	0	0	1	1	1	0
1	1	1	1	0	0	0
0	0	0	0	0	0	0
1	0	0	1	1	1	1
1	1	1	1	0	1	0

	5d	5a	5b	11b	8a	6b
5d	1					
5a	0,6	1				
5b	0,4545	0,8	1			
11b	0,5	0,7	0,5455	1		
8a	0,3333	0,2727	0,2727	0,3	1	
6b	0,6	0,6364	0,5	0,5455	0,4	1
23a	0,5455	0,4615	0,3571	0,3846	0,5	0,7273
6d	0,6364	0,5385	0,4286	0,4615	0,4545	0,8182
25	0,4167	0,4615	0,3571	0,5	0,3636	0,7273
16a	0,4167	0,4615	0,4615	0,6364	0,3636	0,7273
1	0,5	0,5455	0,4167	0,4545	0,4444	0,7
5c	0,6667	0,5455	0,4167	0,4545	0,625	0,7
15a	0,5	0,5455	0,4167	0,4545	0,3	0,7
6a	0,5	0,5385	0,4286	0,4615	0,3333	0,8182
10a	0,3571	0,6154	0,6154	0,4286	0,2143	0,5
10b	0,3077	0,4615	0,4615	0,2857	0,25	0,4615
18	0,4545	0,3846	0,3846	0,3077	0,0769	0,3846
23b	0,4545	0,5	0,5	0,4167	0,1667	0,6364
10c	0,4167	0,4615	0,4615	0,3846	0,25	0,5833
5e	0,3077	0,3571	0,4615	0,2857	0,25	0,5833
14a	0,5	0,5385	0,5385	0,4615	0,2308	0,6667
11c	0,4444	0,3636	0,25	0,4	0,8333	0,5
6c	0,2727	0,4545	0,3333	0,25	0,2	0,4545
10d	0,2727	0,3333	0,3333	0,25	0,2	0,4545
13	0,4	0,6	0,6	0,3636	0,0909	0,3333
19	0,375	0,3	0,3	0,3333	0,125	0,3
15b	0,5556	0,6	0,6	0,3636	0,3333	0,4545
6e	0,4167	0,3571	0,2667	0,3846	0,5	0,5833
12b	0,3333	0,2857	0,3846	0,3077	0,5556	0,3846
3	0,5	0,4167	0,3077	0,4545	0,625	0,5455
12a	0,3636	0,4167	0,3077	0,4545	0,4444	0,5455
21	0,2727	0,3333	0,2308	0,5	0,3333	0,4545
7	0,3846	0,4286	0,5385	0,4615	0,3333	0,3333
26	0,3571	0,3125	0,3125	0,3333	0,3077	0,4
11a	0,3571	0,4	0,4	0,3333	0,4167	0,5
8b	0,4	0,3333	0,3333	0,3636	0,7143	0,4545
9	0,3636	0,4167	0,4167	0,3333	0,4444	0,3077
2	0,25	0,2143	0,2143	0,2308	0,3	0,2143
16b	0,3636	0,4167	0,4167	0,3333	0,3	0,4167
20	0,1818	0,25	0,3636	0,2727	0,375	0,3636
4	0,1818	0,25	0,3636	0,2727	0,2222	0,5
24	0,25	0,3077	0,3077	0,4545	0,4444	0,5455

23a	6d	25	16a	1	5c	15a
1						
0,75	1					
0,5385	0,6154	1				
0,5385	0,6154	0,6667	1			
0,6364	0,5833	0,5	0,5	1		
0,6364	0,7273	0,5	0,5	0,6	1	
0,5	0,5833	0,5	0,5	0,6	0,6	1
0,6154	0,6923	0,75	0,6154	0,5833	0,5833	0,7273
0,375	0,4375	0,375	0,4667	0,5385	0,4286	0,6667
0,3333	0,4	0,3333	0,4286	0,5	0,5	0,5
0,2667	0,3333	0,2667	0,3571	0,4167	0,3077	0,5455
0,5833	0,5385	0,4615	0,5833	0,4167	0,4167	0,5455
0,5385	0,6154	0,4286	0,5385	0,3846	0,5	0,3846
0,5385	0,6154	0,4286	0,5385	0,3846	0,3846	0,5
0,6154	0,6923	0,5	0,6154	0,4615	0,4615	0,4615
0,6	0,5455	0,4545	0,4545	0,5556	0,75	0,4
0,3077	0,3846	0,3077	0,3077	0,3636	0,5	0,5
0,3077	0,5	0,3077	0,4167	0,3636	0,5	0,5
0,2143	0,2857	0,2143	0,2143	0,25	0,25	0,5
0,1667	0,25	0,2727	0,2727	0,2	0,2	0,3333
0,4167	0,5	0,3077	0,3077	0,3636	0,5	0,5
0,5385	0,75	0,5385	0,5385	0,3846	0,6364	0,3846
0,4615	0,4286	0,3571	0,4615	0,3077	0,5455	0,3077
0,6364	0,5833	0,5	0,5	0,4545	0,7778	0,4545
0,5	0,4615	0,5	0,5	0,4545	0,6	0,4545
0,3077	0,3846	0,4167	0,5455	0,3636	0,5	0,5
0,4	0,375	0,2353	0,4	0,3571	0,3571	0,2667
0,4667	0,5333	0,4667	0,375	0,25	0,3333	0,3333
0,4667	0,6429	0,4667	0,4667	0,3333	0,4286	0,4286
0,4167	0,5	0,4167	0,4167	0,3636	0,6667	0,3636
0,2857	0,3571	0,2857	0,2857	0,2308	0,4545	0,2308
0,2	0,3571	0,2	0,2	0,1429	0,3333	0,3333
0,3846	0,4615	0,2857	0,3846	0,3333	0,4545	0,4545
0,3333	0,3077	0,3333	0,4545	0,4	0,2727	0,4
0,3333	0,4167	0,4545	0,6	0,2727	0,2727	0,4
0,5	0,4615	0,5	0,8	0,4545	0,4545	0,3333

6a	10a	10b	18	23b	10c	5e
1						
0,5333	1					
0,4	0,6923	1				
0,4286	0,6154	0,5833	1			
0,5385	0,5	0,4615	0,5	1		
0,5	0,375	0,4286	0,3571	0,7273	1	
0,5	0,4667	0,4286	0,3571	0,7273	0,6667	1
0,5714	0,4375	0,4	0,4286	0,8182	0,9091	0,75
0,4167	0,2857	0,3333	0,1538	0,25	0,3333	0,2308
0,3846	0,4615	0,5455	0,2308	0,3333	0,3077	0,3077
0,3846	0,4615	0,5455	0,3333	0,4545	0,5455	0,5455
0,3846	0,5833	0,3077	0,4545	0,3333	0,2143	0,3077
0,25	0,2308	0,1667	0,3	0,3	0,1667	0,2727
0,3846	0,4615	0,4167	0,3333	0,4545	0,3077	0,4167
0,5	0,2941	0,3333	0,1875	0,3571	0,5385	0,4286
0,3333	0,3125	0,3571	0,2	0,3846	0,4615	0,4615
0,4615	0,3333	0,3846	0,2143	0,4167	0,5	0,3846
0,4615	0,3333	0,3846	0,2143	0,4167	0,5	0,3846
0,3846	0,3571	0,4167	0,2308	0,3333	0,3077	0,3077
0,2941	0,3529	0,2353	0,25	0,3333	0,3125	0,3125
0,4375	0,2632	0,1579	0,2353	0,4	0,4667	0,4667
0,5333	0,4118	0,2941	0,2353	0,3125	0,375	0,4667
0,3846	0,2667	0,3077	0,1429	0,2308	0,3077	0,3077
0,2667	0,25	0,2857	0,1333	0,2143	0,2857	0,2
0,2667	0,25	0,2	0,2143	0,2143	0,2857	0,2857
0,3571	0,4286	0,3846	0,2143	0,3077	0,2	0,3846
0,3077	0,3846	0,3333	0,25	0,3636	0,2308	0,4545
0,4167	0,3846	0,3333	0,25	0,5	0,3333	0,6
0,4615	0,3333	0,3846	0,2143	0,4167	0,5	0,3846

6e	12b	3	12a	21	7	26
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1						
0,5833	1					
0,6364	0,7	1				
0,6364	0,7	0,7778	1			
0,4167	0,3333	0,5	0,5	1		
0,3125	0,4286	0,2667	0,2667	0,2	1	
0,4667	0,4	0,4286	0,3333	0,2667	0,2778	1
0,5714	0,4	0,4286	0,3333	0,3571	0,2778	0,7143
0,5455	0,6	0,6667	0,5	0,5556	0,2857	0,4615
0,3846	0,4167	0,4545	0,3333	0,3636	0,2667	0,4286
0,3846	0,3077	0,3333	0,2308	0,3636	0,1875	0,5385
0,2857	0,3077	0,3333	0,2308	0,3636	0,3571	0,25
0,2308	0,3636	0,2727	0,2727	0,3	0,3077	0,2
0,3333	0,3636	0,2727	0,2727	0,3	0,2143	0,2857
0,5	0,4167	0,4545	0,4545	0,5	0,3571	0,25

11a	8b	9	2	16b	20	4
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1						
0,5833	1					
0,5385	0,6667	1				
0,5385	0,5	0,6	1			
0,4286	0,3636	0,3333	0,2308	1		
0,2857	0,3	0,1667	0,1667	0,2727	1	
0,3846	0,3	0,1667	0,1667	0,4	0,5	1
0,3333	0,3636	0,2308	0,1429	0,2308	0,4	0,4

	M1	Buns	M2	M3	M4	M5	M6	M7	M8	M9	T
1		4						2			
2		3			7	3		4	8	3	
3		3						2			
4				1							
5A				1	12	1			1		
5B					1						
5C					1	2			2		
5D						1			1		
5E									1		
6A				1							
6B				2	1						
6C					1						
6D						1					
6E									1		
7					1						
8A					12	2		1	11	2	
8B									1		
9					1						
10A					2				1		
10B					3				4		
10C					1	1			2		
10D									2		
11A					4	1		3			
11B					1						
11C						1					
12A					3	5		14	11		
12B								1	2		
13					2						
14A					1						
15A					1						
15B					1						
16A					1						
16B					1						
18								7			
19								1			
20								2	1		
21					1				2	22	
23A									3		
23B									1		
24									1		
25											52
26								1			
Total	0	10	0	5	59	18	0	38	56	27	52

READS x genera x sample

Genera	M1	Buns	M2	M3	M4	M5	M6	M7
Acremonium/Sarocladium	1028	72	92	1228	5		9	6
Alternaria		1	1					
Ascosphaera		1						
Aspergillus	3	4				3	3	1
Aureobasidium	10	59	19	28	1			3
Cercospora								
Cladosporium	12	89	34	24	1	0	6	3
Colletotrichum								
Davidiellaceae (fam.)								
Epicoccum								
Eurotium							4	3
Exophiala	1							
Fusarium/Gibberella	5574	2628	236	3326	47	12	31	65
Ganoderma								
Gastrocybe								
Gibellulopsis				1				
Leptodontidium	2	1						
Lulworthia			1					
Mollisia								
Monoblepharella								
Mucor	593	414	1	70	8		6	
Nigrospora	2	2	2	1				
Occultifur	5	7		2				
Papulaspora								
Paracoccidioides							2	2
Paraphaeosphaeria	2							
Penicillium/Talaromyces	51	15	3	22			10	9
Penidiella								
Peniphora			1					
Periconia								
Peyronellaea								
Phialemoniopsis								1
Polyporales (order)			2					
Powellomycetaceae (fam.)			1					
Pyxidiophora								
Rhizopctonia							4	
Rhizopus				1				
Schizophyllum								
Sebacina								10
Skovortzovia				1			1	5
Sphaeronaemella	2							
Sporisorium		1						
Trechispora				2				3
Trichoderma	6			3				
Wallemia	3	1		2			1	
uncultured compost fungus 1F22								
uncultured soil fungus OTU97-907	2							

uncultured ectomycorrhizal fungus C2_EG9	1			3			5	5
uncultured Glomerales B18_41					1			
uncultured fungus ACV_D09-15								1
uncultured Sebaciales 6469								1
uncultured soil fungus NCD_LSU_otu3265								
uncultured fungus HCA_D08-13								
Total	7297	3295	393	4714	63	15	82	118

13

1

1

3 0 5402 90 28 20 0 451 15 30 3 1

Accession

S003508908
S003475732
S003474293
S003475943
S003475818
S003475857
S003474980
S004188945
S004188956
S004189003
S004189004
S004189010
S004189011
S004189012
S000649098
S003832011
S003508851
S003508904
S003440512
S003440552

S003253764
S003253811
S002514002

Total chimeras

Name

uncultured fungus 241FMB6/chimera Cryp. magnus x Candida sp.
uncultured Hanseniaspora MAG_3606/chimera H. uvarum x Hanseniaspora sp.
uncultured Hanseniaspora TBB_313/chimera H. uvarum x S. cerevisiae
uncultured Lachancea FNS_4155/chimera Pichia x H. uvarum
uncultured Saccharomyces HPM_3807/chimera S. cerevisiae x H. uvarum
uncultured Saccharomyces MOV_3934/chimera S. cerevisiae x Starmera sp.
uncultured Saccharomyces NNS_1746/chimera S. cerevisiae x Candida sp.
uncultured yeast 11170328 21/chimera S. cerevisiae x K. marxianus
uncultured yeast 11170371 66/chimera S. cerevisiae x K. marxianus
uncultured yeast ZB05314387 9.19-10/chimera K. marxianus x S. cerevisiae
uncultured yeast ZB05314389 9.19-12/chimera S. cerevisiae x K. marxianus
uncultured yeast ZB05314407 9.19-30/chimera K. marxianus x S. cerevisiae
uncultured yeast ZB05314412 9.19-35/chimera K. marxianus x S. cerevisiae
uncultured yeast ZB05314415 9.19-38/chimera Candida sp. x K. marxianus
uncultured compost fungus 1F31/chimera Debaryomyces sp. x Galactomyces sp.
Saccharomyces cerevisiae/Candida shehatae fusant; CP11
uncultured fungus; 239FMC3/chimera Wick. anomalus x Cryp. magnus
uncultured fungus; 241FMA10/chimera Cryp. magnus x H. uvarum
uncultured fungus; 240FG112011/chimera Cryptococcus x Cryp. magnus
uncultured fungus; 241FC62011/chimera Candida sp. x C. oleophila

uncultured soil fungus; OTU97-881/chimera Acremonium sp. x Mortierella sp.
uncultured soil fungus; OTU97-924/chimera Hymenoscyphus sp. x Cladosporium sp.
uncultured Psathyrella; F4/chimera Psathyrella sp. x Coprinopsis sp.

Reads

2
1
39
11
2
10223
265
11
242
5
15
4
1
19
73
17
19
11
1
3

5
9
1

10964

Fungi and yeast genera represented by less than 1% of the total reads within each sample

	M1	Buns	M2	M3	M4	M5	M6	M7
Alternaria	0	0,01	0,0099	0	0	0	0	0
Ascosphaera	0	0,01	0	0	0	0	0	0
Aspergillus	0,0303	0,0402	0	0	0	0,0198	0,0282	0,0081
Aureobasidium	0,1012	0,5925	0,1873	0,2848	0,0063	0	0	0,0242
Cercospora	0	0	0	0	0	0	0	0
Colletotrichum	0	0	0	0	0	0	0	0
Davidiellaceae	0	0	0	0	0	0	0	0
Epicoccum	0	0	0	0	0	0	0	0
Eurotium	0	0	0	0	0	0	0,0376	0,0242
Exophiala	0,0101	0	0	0	0	0	0	0
Ganoderma	0	0	0	0	0	0	0	0
Gastrocybe	0	0	0	0	0	0	0	0
Gibellulopsis	0	0	0	0,0102	0	0	0	0
Leptodontidium	0,0202	0,01	0	0	0	0	0	0
Lulworthia	0	0	0,0099	0	0	0	0	0
Mollisia	0	0	0	0	0	0	0	0
Monoblepharella	0	0	0	0	0	0	0	0
Nigrospora	0,0202	0,0201	0,0197	0,0102	0	0	0	0
Occultifur	0,0506	0,0703	0	0,0203	0	0	0	0
Papulaspora	0	0	0	0	0	0	0	0
Paracoccidioides	0	0	0	0	0	0	0,0188	0,0162
Paraphaeosphaeria	0,0202	0	0	0	0	0	0	0
Penidiella	0	0	0	0	0	0	0	0
Peniphora	0	0	0,0099	0	0	0	0	0
Periconia	0	0	0	0	0	0	0	0
Phialemoniopsis	0	0	0	0	0	0	0	0,0081
Polyphorales	0	0	0,0197	0	0	0	0	0
Powellomyces	0	0	0,0099	0	0	0	0	0
Pyxidiophora	0	0	0	0	0	0	0	0
Rhizoctonia	0	0	0	0	0	0	0,0376	0
Rhizopus	0	0	0	0,0102	0	0	0	0
Schizophyllum	0	0	0	0	0	0	0	0
Sebacina	0	0	0	0	0	0	0	0,0808
Skovortzovia	0	0	0	0,0102	0	0	0,0094	0,0404
Sphaeronaemella	0,0202	0	0	0	0	0	0	0
Sporisorium	0	0,01	0	0	0	0	0	0
Trechispora	0	0	0	0,0203	0	0	0	0,0242
Trichoderma	0,0607	0	0	0,0305	0	0	0	0
Wallemia	0,0303	0,01	0	0,0203	0	0	0,0094	0
uncultured fungi	0,0303	0	0	0,0305	0,0063	0	0,047	0,0565
Clavispora	0	0,01	0,0099	0	0	0	0	0
Cystofilobasidium	0	0	0	0	0	0	0	0
Eremothecium	0	0	0	0,0305	0	0	0	0
Hannaella	0	0,0703	0	0	0	0	0	0
Kazachstania	0	0	0	0	0	0	0	0
Malassezia	0	0,0201	0	0	0	0	0,0094	0,0081
Metschnikowia	0,0405	0,241	0,0197	0,1526	0	0	0	0
Pseudozyma	0,0303	0,0301	0,0099	0,0305	0	0	0	0
Rhodospiridium	0	0	0	0	0	0	0	0,0242
Sporobolomyces	0	0	0	0	0	0	0	0
Torulaspora	0	0	0	0	0	0	0	0
Yarrowia	0,0303	0,241	0,5225	0,1526	0	0	0,2069	0,1131
Zygoascus	0	0	0	0,0407	0	0	0	0
Zygosaccharomyces	0	0,01	0,0197	0,061	0	0	0,0094	0,0162
uncultured yeast	0,0101	0	0	0,0102	0	0	0	0

M8	M9	T1	T2	T3	T4	T5	T6	T7	T8	T9	Aged
	0	0,352		0	0	0	0	0	0,0161	0	0
	0	0		0	0	0	0	0	0	0	0
	0	0,1466		0	0	0	0	0	0,0161	0	0
	0	0		0	0	0	0	0	0	0	0
	0	0,0147		0	0	0	0	0	0	0	0
	0	0	0,0271		0	0	0	0	0	0	0
	0	0,0147		0	0	0	0	0	0	0	0
	0	0,1027		0	0	0	0	0	0	0	0
	0	0,0147		0,1849	0	0	0	0	0	0	0
	0	0		0	0	0	0	0	0	0	0
	0	0	0,0135		0	0	0	0	0	0	0
	0	0,0147		0	0	0	0	0	0	0	0
	0	0		0	0	0	0	0	0	0	0
	0	0		0	0	0	0	0	0	0	0
	0	0		0	0	0	0	0	0	0	0
	0	0,0147		0	0	0	0	0	0	0	0
	0	0		0	0	0	0	0	0,0242	0	0
	0	0		0	0	0	0	0	0	0	0
	0	0,0293		0	0	0	0	0	0	0	0
	0	0,0147		0	0	0	0	0	0	0	0
	0	0		0	0	0	0	0	0	0	0
	0	0		0	0	0	0	0	0	0	0
	0	0		0	0	0	0	0	0	0	0
	0	0		0	0	0	0	0	0	0,011	0
	0	0		0	0	0	0	0	0	0	0
	0	0,0147		0	0	0	0	0	0	0	0
	0	0		0	0	0	0	0	0	0	0
	0	0		0	0	0	0	0	0	0	0
	0	0		0	0	0	0	0	0	0	0
	0	0		0	0	0	0	0	0	0	0
	0	0		0	0	0	0	0	0	0	0
	0	0,0147		0	0	0	0	0	0	0	0
	0	0		0	0	0	0	0	0	0	0
	0	0		0	0	0	0	0	0,0161	0	0
	0	0		0	0	0	0	0	0	0	0,0109
	0	0		0	0	0	0	0	0	0	0
	0	0		0	0	0	0	0	0	0	0
	0	0		0	0	0	0	0	0	0	0
	0	0		0	0	0	0	0	0	0	0
	0	0		0	0	0	0	0	0	0	0
	0	0,0147		0	0	0	0	0	0	0	0
	0	0,2493	0,0406		0	0	0	0	0,0081	0,011	0
	0	0	0,0406	0,074		0	0	0	0,0484	0	0,0109
	0	0,044		0	0	0	0	0	0	0	0
	0	0		0	0	0	0	0	0	0	0
	0	0,1173		0	0	0	0	0	0	0	0
	0	0		0	0	0	0	0	0,0081	0	0
	0	0	0,0271		0	0	0	0	0,0161	0,011	0
	0	0		0	0	0	0	0	0	0	0
	0	0,0293		0	0	0	0	0	0	0	0
	0	0		0	0	0	0	0	0	0	0
	0	0,0147		0	0	0	0	0	0	0	0
	0	0		0	0,1284		0	0	0,0081	0	0
	0	0,044	0,5007		0	0	0,0113	0	0,9191	0,1211	0,0436
	0	0		0	0	0	0	0	0	0	0
	0,0203	0		0	0	0	0	0	0,0403	0,011	0,0109
	0	0		0	0	0	0	0	0	0	0

Supplementary material Figure legends and Table headlines

Figure 1. Supplementary Material. Rarefaction curves for high-quality D1/D2 26S rDNA reads from chicha samples at 3% distance levels.

Figure 2. Supplementary Material. Relative abundances of filamentous fungi genera and yeast species based on taxonomic assignments of D1/D2 26S rDNA reads from *chicha* fermentation samples. Only taxa accounting for reads below 1% are displayed.

Table 1. Supplementary Material. Data matrix (0/1 table) to test UPGMA based on delta elements profiles from *S. cerevisiae* isolated from chicha.

Table 2. Supplementary Material. Similarity matrix obtained from data matrix (Table 1. Supplementary Material) using the program SIMQUAL in NTSYS package version 2.21p (Exeter Software, USA).

Table 3. Supplementary Material. Number of *S. cerevisiae* isolates distributed by chicha sample and delta elements pattern.

Table 4. Supplementary Material. Number of high-quality D1/D2 26S rDNA reads distributed by taxa and chicha sample. Filamentous fungi and yeast appear in separated spreadsheets.

Table 5. Supplementary Material. Number of reads in our study identified as chimeric sequences. Sequences in RDP fungal database release 11.4 (Accession) labeled as “uncultured” (Name) were blasted against GenBank database in separated 3’ and 5’ regions.

Table 6. Supplementary Material. Percentage of high-quality D1/D2 26S rDNA reads representing less than 1% of the total reads distributed by genera (filamentous fungi or yeast) and chicha sample.

Figure 1Supplementary Material Mendoza et al. 2016

Figure 2 Supplementary Material Mendoza et al. 2016