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Ana L. Freire, Sonia Zapata, Juan Mosquera, Maria Lorena Mejia, Gabriel Trueba

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Bacteria associated to human saliva are major microbial components of Ecuadorian indigenous beers (*chicha*)

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ABSTRACT

Indigenous beers (*chicha*) are part of the indigenous culture in Ecuador. The fermentation process of these beers rely probably on microorganisms from: fermenting substrates, environment and human microbiota. We analyzed the microbiota of artisanal beers (including a type of beer produced after chewing boiled cassava) using bacterial culture and 16S-based tag-encoded FLX amplicon pyrosequencing (bTEFAP). Surprisingly, we found that *Streptococcus salivarius* and S*treptococcus mutans* (part of the human oral microbiota) where among the most abundant bacteria in chewed cassava and in non-chewed cassava beers. We also demonstrated that *S. salivarius* and *S. mutans* (isolated from these beers) could proliferate in cassava mush. *Lactobacillus* sp. was predominantly present in most types of Ecuadorian *chicha*.

INTRODUCTION

The domestication of fermenting bacteria and yeast predated the domestication of animals and plants; ancestral hominids adapted to metabolize alcohol long time before the Neolithic period (*Carrigan et al., 2015*). The organoleptic and psychotropic effects associated with the consumption of accidentally fermented fruits or cereals may have motivated early humans to replicate this process. Additionally, fermentation may have provided unintended benefits as fermenting bacteria may have reduced the risks of foodborne diseases in ancient societies (*Nakamura et al., 2012; Lewus et al., 1991; Fooks & Gibson, 2002; Tesfaye et al., 2011*); it is still unclear whether these microorganisms confer additional health benefits (*McNulty NP, 2011*). The use of alcoholic beverages has played a crucial role in the evolution of human societies (*Joffe, 1998)*, nevertheless, very little is known about the process of domestication and evolution of these fermenting microorganisms (*Libkinda, et al., 2011*).

Many fermenting microorganisms have originated in the environment and food substrates (*Martini, 1993)*, others resemble microorganisms found in the human microbiome suggesting human (skin or intestine) origins (*Agapakis & Tolaas, 2012*); in fact some modern fermented dairy products contain intestinal bacteria (*Walter, 2008*).

Indigenous people from South America (such as Ecuador) prepare a type of beer known as *chicha* which is made with either corn, boiled cassava or the fruit of the palm *Bactris gasipaes* (chonta); some cassava beers include an additional chewing step before the fermentation process. A recent report showed that bacteria present in chewed cassava beers were mainly *Lactobacillus* sp (*Colehour, et al., 2014*). We analyzed the microbial diversity (using culture dependent and culture independent techniques) in different types of Ecuadorian *chicha*.

MATERIALS & METHODS

Sample collection

Four samples of *chicha* (indigenous beer) from two geographical regions of Ecuador (Andean and Amazon regions) were collected. These samples included beer made with both chewed cassava (CC), mushed cassava (MC); mushed chonta (CB) and ground corn (CoB) (Table 1). The samples of CC and MC were purchased from the same household. All these products were obtained from rural communities. None of these beers were pasteurized, nor had they any commercial additives or preservatives. All samples were refrigerated (2 to 8^{o} C) after collection; a 2 mL aliquot of sample was stored at -20^{o}C, for molecular phylotyping.

Plate count of lactic acid bacteria (LAB)

A 20 mL aliquot of each sample was homogenized in 180 mL of a sodium citrate solution (10^{-1} dilution) and ten-fold dilutions were made in saline solution (NaCl 0.9%). One mL of each dilution was inoculated in MRS (pH 5) and M17 (pH 7, 0.5% dextrose) by pour plate method. Two incubation temperatures were used (37^{o}C and 43^{o}C) under aerobic and anaerobic conditions, for 3 to 5 days. The incubation time varied because of the different bacteria present on each product.

Phenotypic characterization

Ten colonies (showing different morphology) were randomly picked from MRS plates from each sample . A subset of colonies showing characteristics of lactic acid bacteria (oxidase negative, catalase negative, Gram positive rods or cocci) was selected for molecular characterization (Table 2); 5 from CC, 6 from MC, 6 from CB and 8 from CoB. Strains were stored at -20^{o}C in MRS or M17 broth with 20% of glycerol.

Genotypic characterization of bacterial colonies

DNA was extracted from one colony using DNAzol Reagent (Life Technologies, 2001) following manufacturer instructions and the DNA was stored at -20°C until used. The 16S ribosomal gene was amplified in 25ul containing: 1X PCR buffer, 2.5mM $MgCl_2$, 0.25mM dNTP's, 0.2uM 27F primer (5'-AGAGTTTGATCCTGGCTCAG-3'), 0.2uM 1492R primer (5'-GGTTACCTTGTTACGACTT-3') (Martin, et al., 2001), 0.5U GoTaq Flexi DNA polymerase (Promega, Madison), 5uL of sample DNA and Milli-Q water. The times and temperatures used for the amplification were: melting (94°C, 1 minute), annealing (56°C, 30 seconds), elongation (72°C, 30 seconds), this routine was repeated for 30 cycles, and final extension (72°C, 10 minutes). Amplicons were subjected to gel electrophoresis (1% agarose gel), sequenced at Functional Biosciences (Madison, WI) and DNA sequences analyzed using Seqmatch (Ribosomal Database Project http://rdp.cme.msu.edu/) and submitted to GenBank; the accession numbers are: KT722809 to KT722833).

High throughput sequencing analysis

In order to complement the culture-based protocols, we investigated the microbial diversity using FLX amplicon pyrosequencing. DNA was extracted from all beer samples using DNeasy Plant Mini kit (Qiagen) following manufacturer's protocols, but instead of using AE buffer for elution, we used same volume of PCR Milli-Q water. DNA samples from four types of beer were sent to CD Genomics (NY, USA), for 16S-based phylotyping. DNA was subjected to bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) using primers 939F-5'TTGACGGGGGCCCGCAC3' and 1492R-5'TACCTTGTTACGACTT3'. For fungal sequences we used ITSF- 5'CTTGGTCATTTAGAGGAAGTAA3'. Resulting sequences (minimum length = 250 nucleotides) were trimmed and quality scored using USearch (http://drive5.com/); chimeras were detected using UCHIIME (http://drive5.com/) in de novo

mode and were compared using BLASTn to a ribosomal database. Identity values were used to make assignments to the appropriate taxonomic levels: greater than 97% identity were resolved at the species level and between 95% and 97% at the genus level. The number of bacterial sequences we obtained were: 2,965 readings for CC, 3,320 for MC, 3,046 for CB and 15,623 for CoB. For fungi we obtained 6,763 readings from CC, 6,925 from MC and 6,558 from CB. We did not carry out fungi analysis of CoB. All sequences were submitted to Sequence Read Archive and accession numbers are: SRP070493, SRS1299611, SRX1612367,SRR3202831, SRS1299612, SRX1612366, SRR3202830, SRS1299613, SRX1612365, SRR3202829, SRS1310202, SRX1600290, SRR3187397, SRS1310203, SRX1600289, SRR3187396, SRS1310204, SRX1600288, SRR3187395, SRS1310207, SRX1612364, SRR3202828, SRS1310208, SRX1600292, and SRR3202832.

Streptococcus salivarius and *Streptococcus mutans* growth in cassava solution

To rule out the possibility of *S. salivarius* or *S. mutans* contamination, one colony of a pure culture of each bacteria (obtained from beers) was diluted in 25mL of sodium citrate (2%) separately. Subsequently, 1 mL of this cell suspension was used to inoculate tubes containing 9mL of sterile (autoclaved) chewed cassava solution (10%) and incubated at 37°C under anaerobic conditions. A 100 µL aliquot from each incubated tube was extracted and plated in M17 (this was done by triplicate) at 0, 24, 48 and 72 hours of inoculation. Results from each day were compared to determine the ability of these bacteria to grow in chewed cassava solution.

Statistical analysis

We used Mann-Whitney U test to test whether *S. salivarius* and *S. mutans* were able to grow in cassava solution. Shannon indices were calculated using the formula $H = -\sum p_i \log(p_i), p_i$

being the relative frequency of the abundance of each species found. Principal component analysis (PCA) of the bacterial species and abundance of the four beverages was performed using the software SPSS v21 (IBM Corp, Armonk, NY).

RESULTS

Characterization of bacterial isolates

Twenty-five bacterial isolates (cultured from the 4 beer types) were characterized by 16s rDNA sequencing showing 99% to 100% identity when compared with GenBank sequences (Table 2). The predominant bacterial species in all beers were *Lactobacillus fermentum* (16%), *Lactococcus lactis* (16%), *Leuconostoc mesenteroides* (16%), and *Streptococcus salivarius* (16%); followed by *Lactobacillus plantarum (*8%*), Weissella confusa* (8%), *Lactobacillus casei* (4%*), Lactobacillus pantheris* (4%*), Lactobacillus parabuchneri* (4%*), Lactobacillus paracasei* (4%) and *Streptococcus mutans* (4%). The most diverse bacterial composition (using culture-dependent techniques) found in CoB (6 bacterial species), followed by the CC (5 bacterial species), CB (3 bacterial species) and MC (2 bacterial species). Intriguingly cassava beers contained human salivary bacteria: both CC and MC, had *Streptococcus salivarius* while CC had also *S. mutans* (Table 2).

High throughput sequencing analysis

The beer with greater diversity was CC (31 bacterial species), followed by CoB (26 bacterial species), CB (21 bacterial species), MC (20 bacterial species). The predominant bacterial species in CC were *Lactobacillus* spp. (40.9%) followed by human microbiota bacteria: *Streptococcus salivarius* (31.94%), *Streptococcus parasanguinis* (5.41 %), *Streptococcus pneumoniae* (3.65%). The most prevalent bacteria in MC were *Streptococcus* spp. (83%) followed by *Lactococcus* sp. (9.32%); the majority of streptococci have been described as part of the human microbiota: *Streptococcus salivarius* (65%), *Streptococcus pasteurianus* (7.74%), and *Streptococcus parasanguinis* (3.47%). The most prevalent bacteria in CB were *Weissella confusa* (46%), *Weissella* sp. (20%), and *Lactococcus lactis* (9%). The dominant bacteria in CoB were

142 *Weissella* sp. (19%) and *Lactobacillus plantarum* (12.5%), *Lactococcus garviae* (2.76%)
143 *Lactobacillus brevis* (2.5 %) (Table 3). The dominant fungal species present in different beers
144 analyzed was very similar; *Saccharomyces cerevisiae* was the most abundant comprising 92% of all
145 the taxa detected (Table 4).

146 **Growth of *S. salivarius* and *S. mutans* in cassava solution**

147 *Streptococcus salivarius* (Figure 1) *and S. mutans* (Figure 2) grew in chewed cassava
148 solution. After 48 hours of culture (*S. salivarius*) and 72 hours (*S. mutans*), bacterial counts went
149 down.

150 **Diversity estimations**

151 CC was the beverage with the most species diversity (H=1.06, E= 0.71), followed by CoB
152 (H=0.94, E=0.66), CB (H=0.71, E=0.54), and MC (H=0.59, E=0.45). The evenness values
153 followed the same pattern and suggest that CC is also the most heterogeneous in terms of species
154 (*Hayek, 2010; Pielou, 1966*).

155 **Principal component analysis**

156 The type of beer (fermenting substrate) accounted for 90.4% of the bacterial species
157 variability and cassava beers had more similar bacterial composition and abundance than the
158 other types of beer; interestingly CB and CoB also showed similarity (Figure 3).

159 **DISCUSION**

160 Our study found higher bacterial diversity in beer that contained human saliva (Tables 2
161 and 3); therefore, saliva may not only speed up the fermentation process (by providing amylases
162 as suggested by Henkel, 2005) but also may offer an additional bacterial inoculum which may
163 favor this process. This finding may provide additional explanation for the adoption of such a
164 peculiar process in the beer´s manufacture.

165 Our study also demonstrate the presence of oral streptococci such as *S. salivarius, S.*
166 *mutans, S. parasanguinis* in cassava beers; these bacteria may thrive on carbohydrates present in
167 the oral cavity after starchy meals (*Moye, et al., 2014; Burne, et al., 1998*). Oral bacteria *S.*
168 *salivarius* and *S. mutans* were cultured from cassava chicha (with saliva and without saliva) in
169 large numbers and were shown to grow in mushed cassava under laboratory conditions. Oral
170 bacteria in beer without human saliva may indicate contamination of fermenting containers (or
171 utensils). Fermenting bacteria are known to produce biofilm in containers (*Kebede et al., 2007*)
172 and both types of cassava beers were obtained from the same household, and probably they use
173 the same pots for both type of beers. It is possible that some strains of *S. salivarius* from these
174 beers may be adapting to the fermentation process; *Streptococcus thermophilus*, a bacteria used
175 as starter in yogurt *(Burton, et al., 2006*) may have evolved from *S. salivarius* (*Hols, et al., 2005*).
176 Future studies should investigate the prevalence of *S. salivarius* in larger number of cassava
177 *chichas* from other locations and find out whether the strains of *S. salivarius* isolated from beers
178 are different from those isolated from human saliva.

179 A recent study failed to detect *S. mutans* and *S. salivarius* in *chicha* prepared with chewed
180 cassava in Ecuador (*Colehour, et al., 2014)*. The disagreement between both studies may result
181 from differences in samples in both studies; Colehour, et al., 2014 collected beers that were

182 fermenting for 4 days while we collected samples that were fermenting for 3 days. Beer microbiota
183 changes overtime *(Steinkraus, 2006)* and in the case of *S. mutans* and *S. salivarius* we observed a
184 sharp increase and decline in bacterial populations in 24 hours (Figures 1 and 2). Unlike Colehour,
185 et al., 2014, we also carried out bacterial cultures.

186 Reduction on streptococci populations may be due to the consumption of all the nutrients,
187 accumulation of toxic metabolites, autolysis. (*Dufour & Lévesque, 2013)*. Also, these bacteria are
188 known to form biofilm (*Ajdic, et al., 2002; Li, et al., 2002*) which may change bacterial location
189 and reduction of planktonic cells. Additionally, unlike our study Colehour, et al., 2014 found
190 predominance of *L. reuteri* which is known to antagonize *S. salivarius (Nikawa, et al., 2004;*
191 *Corby, et al., 2005)*. Similar to previous studies (*Colehour, et al., 2014; Elizaquivel, et al., 2010;*
192 *Puerari, et al., 2015)*, *Lactobacillus* was a dominant genus of lactic bacteria in chicha found in
193 both culture dependent and independent assessments.

194 Our study complements previous microbiological analysis carried out in *chicha* and shows
195 for the first time the potential adaptation of *S. salivarius, S. mutants* (and possibly other
196 streptococci from the human upper respiratory tract) to grow in cassava mush. The study not only
197 shows how bacteria from human microbiota may adapt to artisanal fermentative processes but also
198 shows that chewed chicha may potentially transmit human pathogens such as *S. mutans,* one of
199 the causative agents of dental plaque and cavities *(Loesche, 1986*); *Streptococcus mutans* can be
200 transmitted person to person probably through saliva (*Baca et al., 2012*). This is especially relevant
201 because these types of beers are consumed as early as 2 or 3 days after preparation.

202 The main limitation of our study was the low number of samples analyzed of each beer.
203 However this limitation does not invalidate the main findings of this study. Additionally, the

culture medium (MRS) is not suitable to culture *Lactobacillus* from cereals (*Minervini, et al., 2012*), therefore we may have underestimated the bacterial diversity in these beers.

206

207 **ACKNOWLEDGEMENTS**

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210

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FIGURES

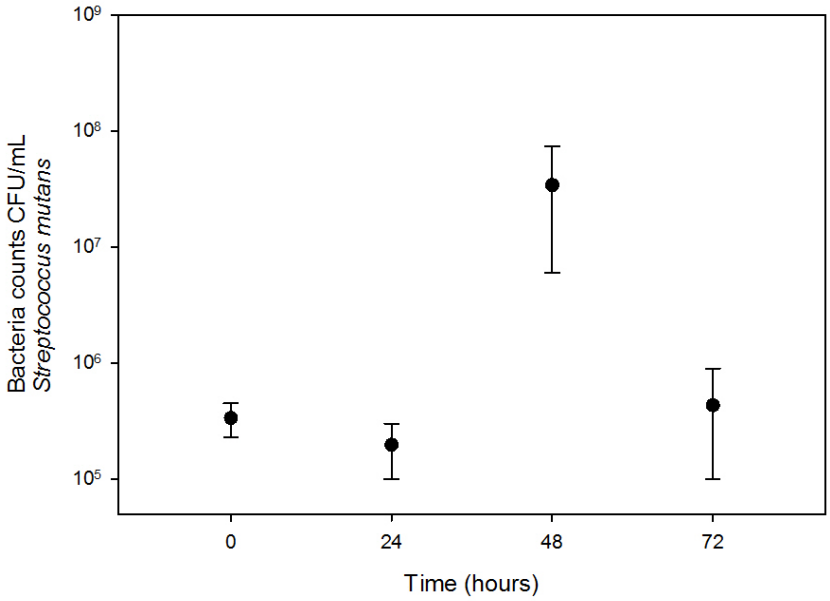

Figure 1. Growth of *S. salivarius* in sterile chewed cassava solution. There is a significant increase in CFU (Mann-Whitney U test) at the 24 hours of incubation compared with those at inoculation time (0 hours).

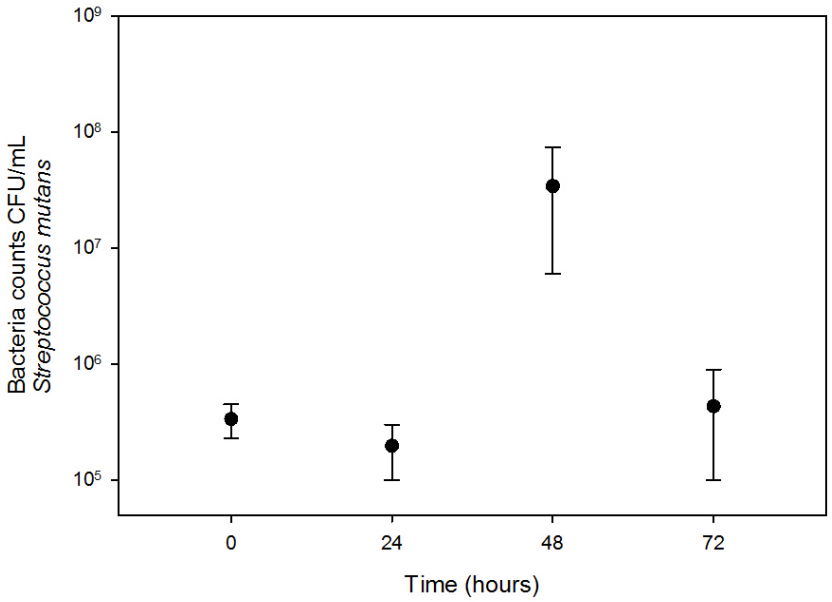

Figure 2. Growth of *S. mutans* in chewed cassava solution. There is a significate increase in CFU (Mann-Whitney U test) at the 48 hour of incubation compared with those at the inoculation time (0 hours).

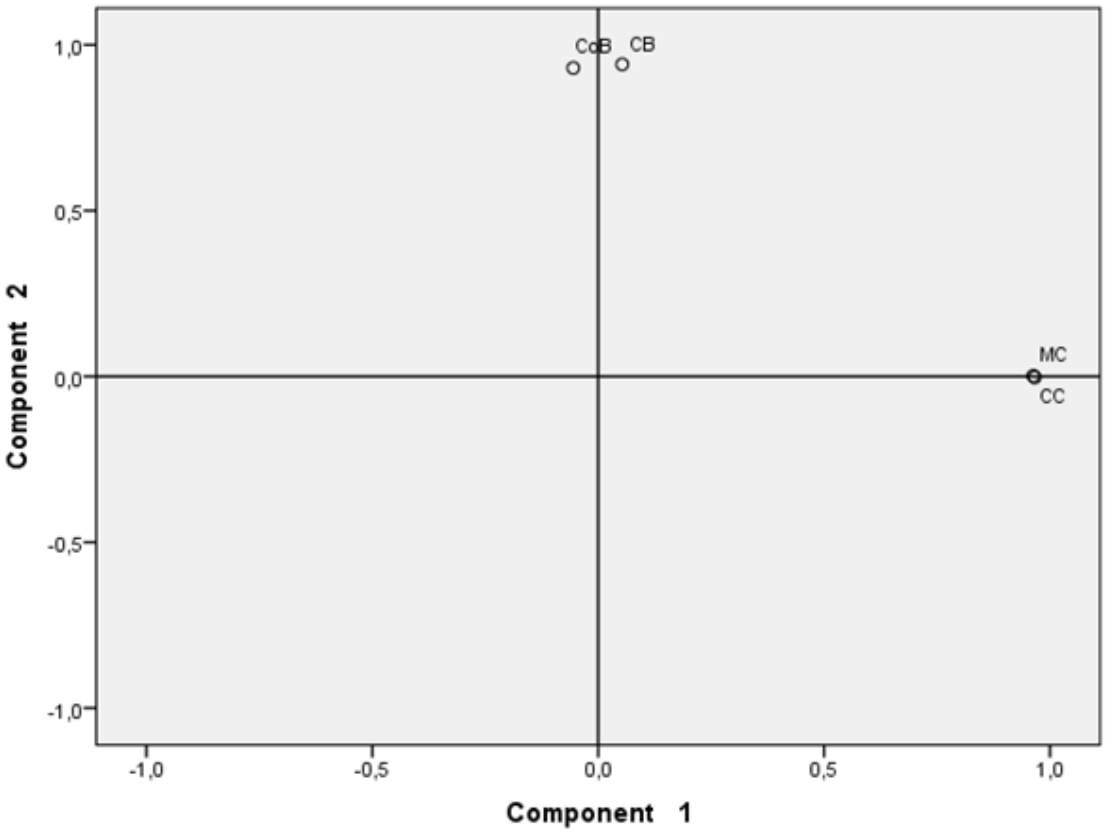

Figure 3. **Principal component analysis of beers' microbiota**. Beers made with cassava (MC and CC) formed a cluster different from the cluster formed by beers made with either chonta (CB) or corn (CoB). Each pair of beverages that form a group share a similar bacterial species profiles and abundance.

344 **Table 1. Description and site of collection of the different types of indigenous beers**
345 **analyzed.**

Main ingredient	Substrate Scientific name	Geographical region	Site of collection	Time of fermentation
Chewed cassava	*Manihot esculenta*	Amazon	Puyo	3 days
Mushed cassava	*Manihot esculenta*	Amazon	Puyo	3 days
Chonta	*Bactris gasipaes*	Amazon	Tena	2 days
Corn (jora)	*Zea mays*	Highlands	Pifo	2 days

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Table 2. Bacteria isolated from the four beer samples. All the 25 strains were obtained by bacterial cultures in MRS and M17 and 16s ribosomal gene from colonies was amplified and sequenced.

Sample	Isolate ID	Culture Media	Growth condition	Identification (16S)
Chewed cassava beer	25 A2	MRS	Anaerobic	*Leuconostoc mesenteroides*
	25 C2	MRS	Aerobic	*Lactobacillus fermentum*
	25 E2	M17	Anaerobic	*Streptococcus mutans*
	25 F1	M17	Aerobic	*Lactococcus lactis*
	25H1	M17	Aerobic	*Streptococcus salivarius*
Mushed cassava beer	26 A1	MRS	Anaerobic	*Lactobacillus fermentum*
	26 B1	MRS	Anaerobic	*Lactobacillus fermentum*
	26 C2	MRS	Aerobic	*Lactobacillus fermentum*
	26 E2	M17	Anaerobic	*Streptococcus salivarius*
	26 F2	M17	Anaerobic	*Streptococcus salivarius*
	26 G1	M17	Aerobic	*Streptococcus salivarius*
Chonta beer	27 A1	MRS	Anaerobic	*Lactobacillus plantarum*
	27 B1	MRS	Anaerobic	*Weissella confusa*
	27 C1	MRS	Aerobic	*Weissella confusa*
	27 E1	M17	Aerobic	*Lactococcus lactis*
	27 F2	M17	Anaerobic	*Lactococcus lactis*
	27 G2	M17	Aerobic	*Lactococcus lactis*
Corn beer	61 B2	MRS	Anaerobic	*Lactobacillus casei*
	61 G1	M17	Anaerobic	*Leuconostoc mesenteroides*
	61 G2	M17	Anaerobic	*Lactobacillus plantarum*
	61 H1	MRS	Anaerobic	*Lactobacillus parabuchneri*
	61 I1	MRS	Anaerobic	*Lactobacillus paracasei*
	61 J1	MRS	Anaerobic	*Lactobacillus pantheris*
	61 K1	M17	Anaerobic	*Leuconostoc mesenteroides*
	61 L1	M17	Anaerobic	*Leuconostoc mesenteroides*

371 **Table 3. Most predominant bacterial species (abundance of more than 0.1%) found by**
372 **pyrosequencing analysis of samples from 4 types of chicha.** Chewed cassava (CC), mushed
373 cassava (MC), chonta (CB) and corn (CoB). Numbers indicate percentages and "+" indicates that
374 bacterium recovered in culture.
375

Bacterial species	CC	MC	CB	CoB	Cultured	Possible origins
Bacillus amyloliquefaciens	0.0	0.5	0.0	0.00	-	Environment
Carnobacterium maltaromaticum	0.0	0.0	1.0	0.1	-	Environment
Enterobacter asburiae	0.5	0.0	0.0	0.0	-	Environment
Enterobacter cancerogenus	0.5	0.0	0.0	0.0	-	Environment
Enterobacter sp	1.3	0.0	0.1	0.0	-	Environment
Fructobacillus sp	0.0	0.0	0.0	3.8	-	Vegetables
Gluconacetobacter intermedius	0.0	0.0	0.0	0.6	-	fermented food
Kluyvera ascorbate	0.4	0.0	0.0	0.0	-	Human gut, food
Lactobacillus brevis	8.4	0.1	2.5	0.6	-	Environment, gut
Lactobacillus camelliae	0.0	0.0	0.0	7.3	-	Environment, gut
Lactobacillus casei	0.0	0.0	0.0	3.1	+	Environment, gut
Lactobacillus delbrueckii	8.0	0.0	0.0	0.0	-	Environment, gut
Lactobacillus fermentum	6.5	3.8	0.0	0.0	+	Environment, gut
Lactobacillus harbinensis	0.0	0.0	0.0	2.1	-	Vegetables
Lactobacillus manihotivorans	1.8	0.0	0.0	0.0	-	Vegetables
Lactobacillus parabuchneri	0.0	0.0	0.0	1.4	+	Oral microbiota
Lactobacillus paracasei	0.0	0.0	0.0	8.6	+	Environment, gut
Lactobacillus paracollinoides	0.0	0.0	0.0	16.0	-	Environment, gut
Lactobacillus plantarum	10.8	0.0	12.4	0.1	+	Environment, gut
Lactobacillus sp	3.4	0.0	0.7	1.3	-	Environment, gut t
Lactobacillus vaccinostercus	1.2	0.0	0.2	0.0	-	Environment, gut
Lactococcus garviae	0.0	0.0	2.8	0.0	-	Fermented food
Lactococcus lactis	2.1	0.0	8.9	0.0	+	Environment, gut
Lactococcus sp	0.2	9.3	1.0	0.2	-	Gut
Leuconostoc citreum	0.0	1.5	1.2	0.0	-	fermented food
Leuconostoc lactis	1.7	0.1	0.2	0.8	-	Environment
Leuconostoc sp	0.0	0.0	0.1	4.6	-	Vegetables
Oenococcus kitaharae	0.0	0.0	0.0	1.2	-	Vegetables
Serratia sp	1.0	0.0	0.0	0.0	-	Environment
Streptococcus gallolyticus	0.0	0.5	0.0	0.0	-	Oral microbiota
Streptococcus oralis	1.4	0.2	0.0	0.0	-	Oral microbiota
Streptococcus parasanguinis	5.4	3.5	0.0	0.0	-	Oral microbiota
Streptococcus pasteurianus	0.0	7.7	0.0	0.0	-	Human gut
Streptococcus pneumoniae	3.6	0.5	0.0	0.0	-	Human nasopharynx
S. pseudopneumoniae	0.5	0.0	0.0	0.0	-	Human nasopharynx
Streptococcus salivarius	32.0	65.0	0.0	0.0	+	Oral microbiota
Streptococcus sp	2.5	2.3	0.1	0.0	-	Human microbiota

Streptococcus thermophilus	1.2	2.59	0.0	0.0	-	Vegetables
Streptococcus vestibularis	0.4	0.8	0.0	0.0	-	Oral microbiota
Weissella cibaria	0.1	0.0	0.9	0.9	-	Vegetables
Weissella confusa	0.5	0.1	45.9	25.3	+	Vegetables
Weissella paramesenteroides	0.5	0.0	0.1	0.0	-	Environment
Weissella sp	0.2	0.3	19.8	19.4	-	Vegetables

Table 4. Most predominant fungal species found by pyrosequencing analysis of samples from 3 types of *chicha*. Chewed cassava (CC), mushed cassava (MC) and chonta (CB). The numbers indicate percentages.

Fungal species	CC	MC	CB	Possible origins
Saccharomyces cerevisiae	92.533	92.023	92.033	Vegetables
Penicillium citrinum	0.03	0.021	0.062	Soil
Debaryomyces hansenii	0.636	0.547	0.549	Sea water
Hanseniaspora uvarum	0.044	0.056	0.075	Vegetables
Wallemia muriae	0.118	0.115	0.137	Salty water
Wallemia sp	1.316	1.701	1.602	Salty water
Aspergillus sp	0.089	0.047	0.032	Soil
Pichia kudriavzevii	1.05	1.5	1.32	Vegetables
Aspergillus versicolor	0.104	0.138	0.135	Soil
Pichia burtonii	0.118	0.123	0.107	Vegetables
Hyphopichia burtonii	0.089	0.067	0.073	Starch substrates
Cyberlindnera sp	0.532	0.54	0.545	Waste deposits
Pichia sp	0.044	0.04	0.054	Soil
Saccharomyces bayanus	0.104	0.132	0.096	Vegetables
Galactomyces sp	3.149	2.908	3.133	Rumen, fermented food
Pichia fermentans	0.044	0.042	0.047	Vegetables

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