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

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Subjects: Food Microbiology, Anthropology

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ABSTRACT

Indigenous beers (*chicha*) are part of the indigenous culture in Ecuador. The fermentation process of these beers relay 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*.

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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 to 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 reduce the risks of food borne 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. We analyzed the microbial diversity (using culture and culture-independent techniques) in different types of Ecuadorian *chicha*.

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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 either chewed cassava (CC), mushed cassava (MC), mushed chonta (CB) and grinded corn (CoB) (Table 1). The samples of CC and MC were purchased form 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 8º C) after collection; a 2 mL aliquot of sample was stored at -20ºC, 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 (37ºC and 43ºC) 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 to twenty colonies (showing different morphology) were randomly picked from each sample. Six to ten colonies that had the characteristics of lactic acid bacteria (oxidase negative, catalase negative, Gram positive rods) were isolated for further characterization. Strains were stored at -20ºC in MRS or M17 broth with 20% of glycerol.

Genotypic characterization of bacterial colonies

From each colony the 16S ribosomal gene was amplified and sequenced. DNA extraction was performed with the DNAzol Reagent (Life Technologies, 2001). One pure

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colony was needed, and all the steps were followed as recommended by the manufacturer. 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: denaturation (94°C, 1 minute), annealing (56°C, 30 seconds), elongation (72°C, 30 seconds), extension (72°C, 30 seconds), final extension (72°C, 10 minutes); this routine was repeated for 30 cycles. Amplicons were analyzed by gel electrophoresis in a 1% agarose gel, sequenced (Functional Biosciences) and sequences compared to BLAST GenBank Database (GenBank accession numbers from 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.

***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

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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. Pielou's evenness index was calculated using the formula $E = H/\log(n)$, n being the number of species found in each beverage (*Hayek & Buzas, 2010; Pielou, 1966*).

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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 confuse* (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 *Lactocacillus* 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

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dominant bacteria in CoB were *Weissella* sp. (19%) and *Lactobacillus plantarum* (12.5%), *Lactococcus garviae* (2.76%) *Lactobacillus brevis* (2.5 %) (Table 3).

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

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

Diversity estimations

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

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DISCUSION

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

Our study also demonstrate that oral streptococci such as *S. salivarius, S. mutans, S. parasanguinis* in cassava beers; these bacteria may thrive on carbohydrates present in the oral cavity after starchy meals (*Moye, et al., 2014; Burne, et al., 1998*). Oral bacteria *S. salivarius* and *S. mutans* were cultured from chicha made with cassava in which these bacteria were present in large numbers. Oral bacteria in beer without human saliva may indicate contamination of fermenting containers (and other utensils) or presence of bacterial biofilms in containers, as found in other bacteria from African traditionally fermented milk (*Kebede et al., 2007*). Additionally both chewed and non-chewed chichi was obtained from the same household. Oral streptococci proliferate in sterile cassava mush as showed in our experiments, Figures 1 and 2.

Streptococcus salivarius is a homofermentative and is closely related to *Streptococcus thermophilus,* which is one of the microorganisms mostly used as starter culture *(Burton, et al., 2006*); *S. mutans* is one of the principal causative agents of dental plaque and dental cavities *(Loesche, 1986*) ; *Streptococcus mutans* can be transmitted person to person (*Baca et al., 2012*)

A recent study failed to detect *S. mutans* and *S. salivarius* in *chicha* prepared with chewed cassava in Ecuador (*Colehour, et al., 2014).* The disagreement between both studies may result from differences in samples in both studies; Colehour, et al., 2014 collected beers that were fermenting for 4 days while we collected samples that were fermenting for 3 days.

Beer microbiota changes overtime *(Steinkraus, 2006)* and in the case of *S. mutans* and *S. salivarius* we observed a sharp increase and decline in bacterial populations in 24 hours (Figures 1 and 2). Unlike Colehour, et al., 2014, we also carried out bacterial cultures.

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

Our study complements previous microbiological analysis carried out in *chicha* and shows for the first time the potential adaptation of *S. salivarius, S. mutants* (and possibly other streptococci from the human upper respiratory tract) to grow in cassava mush. The study not only shows how bacteria from human microbiota may adapt to artisanal fermentative processes but also shows that chewed chicha may potentially transmit human pathogens. The main limitation of our study was the low number of samples analyzed of each beer. However this limitation does not invalidate the main findings of this study.

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Gabriel Trueba and Sonia Zapata designed the experiments and reviewed the paper.
- Ana L. Freire performed the experiments and prepared the paper.
- Juan Mosquera performed some experiments.

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287 **FIGURES**

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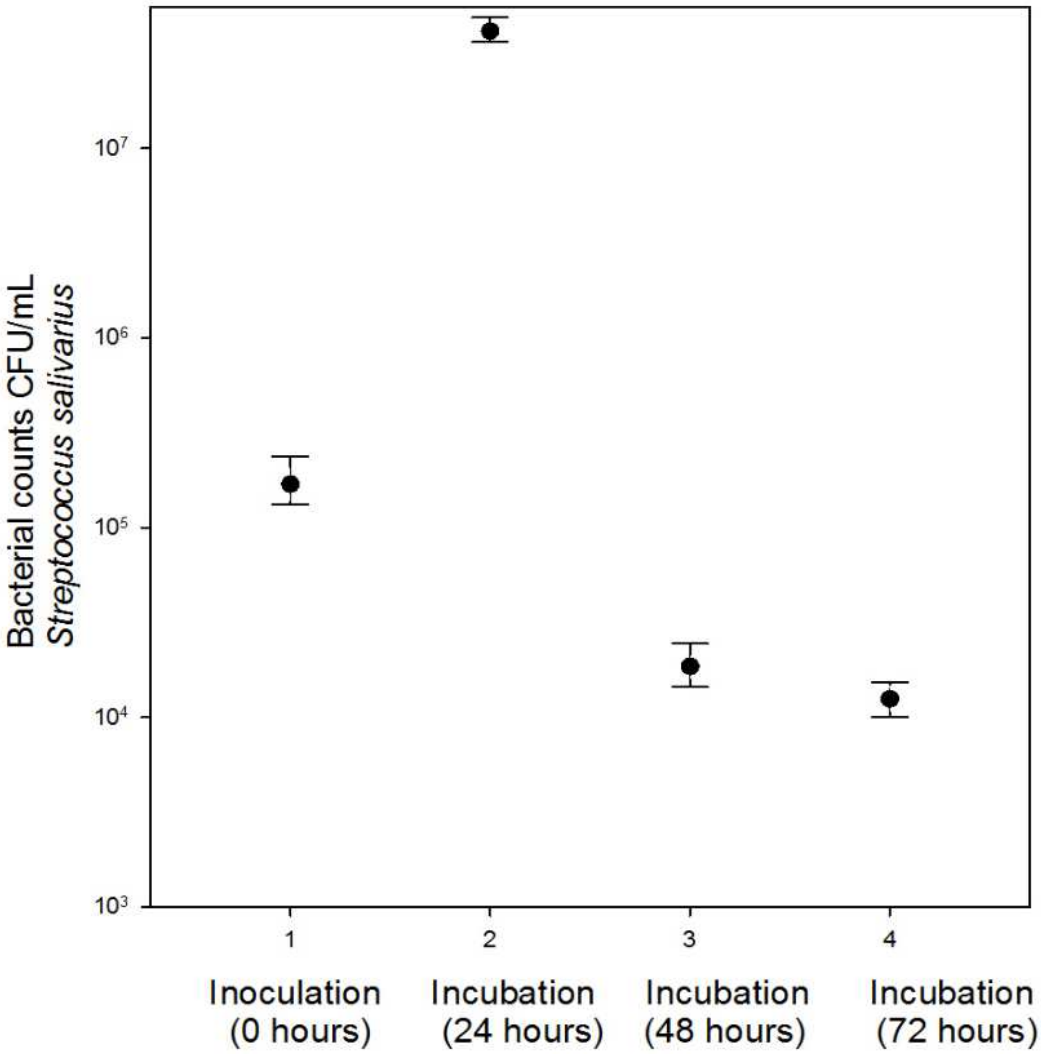

289

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

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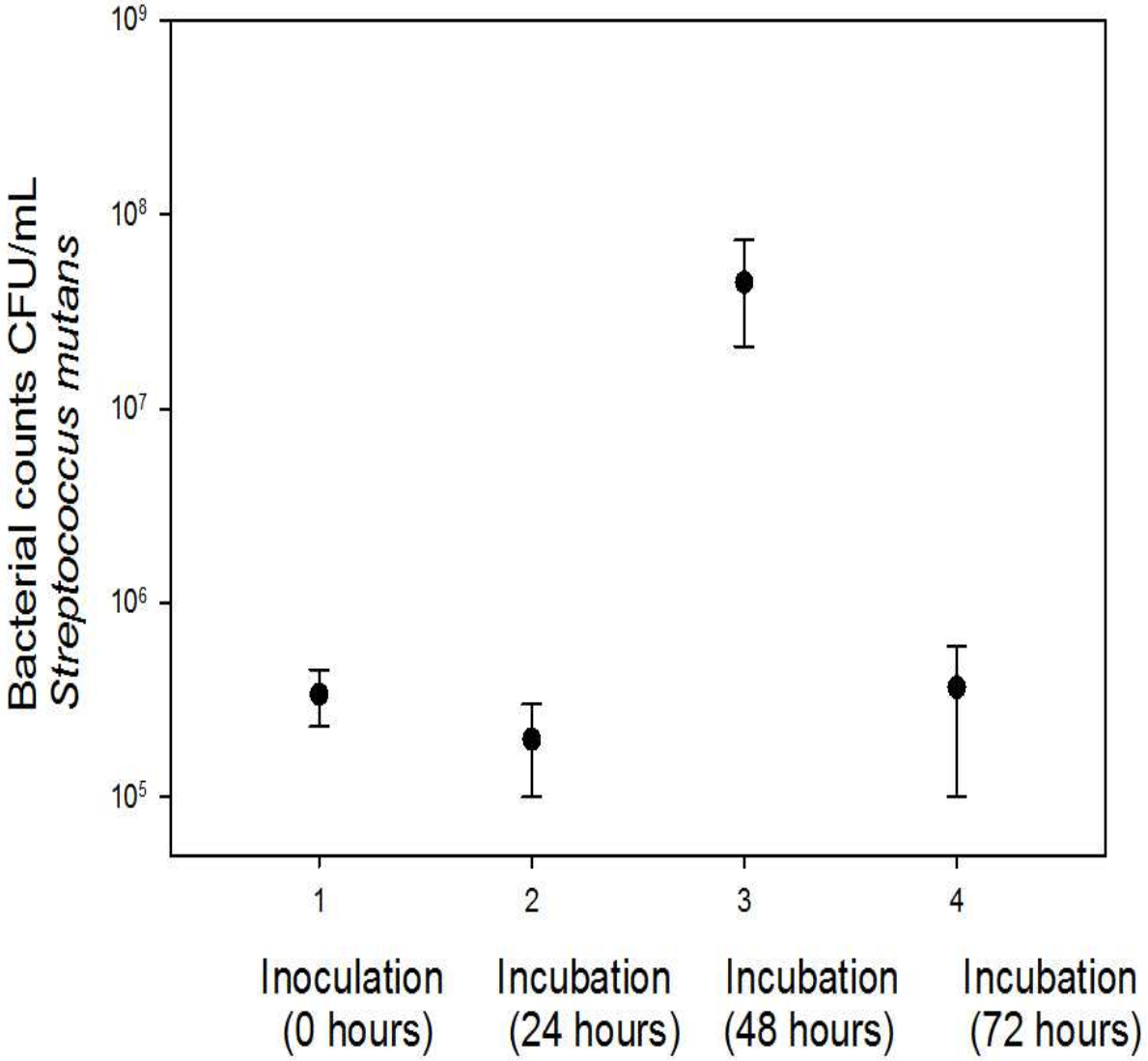

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305 **Figure 2. Growth of *S. mutans* in chewed cassava solution.** There is a significate increase in

306 CFU (Mann-Whitney U test) at the 48 hour of incubation compared with those at the

307 inoculation time (0 hours).

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Table 1. Description and site of collection of the different types of indigenous beers 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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328 **Table 2. Bacteria isolated from the four beer samples.** All the 25 strains were obtained by
329 bacterial cultures MRS and M17 and 16s ribosomal gene from colonies was amplified and
330 sequenced.

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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*

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

Bacterial species	CC	MC	CB	CoB	Cultured	Possible origin
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

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Weissella cibaria	0.1	0.0	0.9	0.9	-	Vegetables
Weissella confuse	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

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