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Streptococcus salivarius, bacteria associated to human saliva is a major component of indigenous beer (*chicha*) from Ecuador

Ana Lucía Freire Vélez

Sonia Zapata Mena, PhD., Directora de Trabajo de Titulación

Trabajo de titulación presentado como requisito para la obtención del título de Magister en Microbiología

Quito, julio de 2015

UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

COLEGIO DE POSGRADOS

HOJA DE APROBACIÓN DE TRABAJO DE TITULACIÓN

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Ana Lucía Freire Vélez

Sonia Zapata Mena, PhD.,
Directora de Trabajo de Titulación _____

Gabriel Trueba Piedrahita, PhD.,
Director del Programa de Microbiología _____

Stella de la Torre, PhD.,
Decana del Colegio de Ciencias Biológicas y Ambientales _____

Víctor Viteri, PhD.,
Decano del Colegio de Posgrados _____

Quito, julio de 2015

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Firma del estudiante: _____

Nombres y apellidos: Ana Lucía Freire Vélez

Código: 00113957

Cédula de Identidad: 0103329959

Lugar y fecha: Quito, julio de 2015

RESUMEN

Las cervezas andinas (chichas) juegan un papel importante en los eventos culturales de comunidades indígenas del Ecuador. En general, la producción de este tipo de cerveza es artesanal y no requiere de la adición de fermentos comerciales liofilizados. El proceso de fermentación es espontáneo, y depende principalmente de: microorganismos propios del medio ambiente, vasijas y recipientes de lotes anteriores; y en ciertos casos de la saliva humana (chichas masticadas). En el presente trabajo se analizó el microbioma de cervezas artesanales utilizando tanto cultivo tradicional como pirosecuenciamiento (bTEPAF). Por medio de las dos técnicas, fue posible determinar la predominancia del género *Lactobacillus* en las cuatro muestras de chicha analizadas. *Streptococcus salivarius* y *Streptococcus mutans*, (componentes de la microbiota normal de la boca) fueron identificados en muestras correspondientes a chichas de la región Amazónica. En base a los experimentos realizados, se pudo demostrar que *S. salivarius* y *S. mutans* pueden proliferar en una solución de yuca.

Palabras clave: chicha*, Streptococcus salivarius*, bebida fermentada artesanal, *Streptococcus mutans*, yuca fermentada, bacterias ácido lácticas, saliva, chicha masticada.

ABSTRACT

Indigenous beers (chicha) play an important role in cultural events of indigenous people from Ecuador. In general, all the Ecuadorian production of indigenous beer is artisanal and it does not include the addition of any starter cultures or lyophilized ferments. The fermentation depends mostly on microorganisms from the environment (including vessels from previous batches of fermented beverage). We analyzed the microbiota of artisanal beers using bacterial cultures and 16S-based tag-encoded FLX amplicon pyrosequencing (bTEFAP). As previous reports we found that *Lactobacillus* sp. is predominantly present in most types of Ecuadorian chicha; however we found that *Streptococcus salivarius* and S*treptococcus mutans* (part of the human mouth microbiota) were also part of microbiota of chewed beers from the Amazon region. We also demonstrated that *S. salivarius* and *S. mutans* could proliferate in cassava mush.

Key words: indigenous beer, *Streptococcus salivarius*, artisanal fermented beverages, *Streptococcus mutans*, fermented cassava, lactic acid bacteria, saliva, chewed indigenous beer.

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PART I

GENERAL INTRODUCTION

History of fermented food

The first records of fermented foods appeared in the Middle East, and date back to 6,000 years before Christ. Fermentation from the beginning of the civilization was probably used to preserve food or to produce different flavors or alcohol. During the 19th century, people began to understand the process of fermentation and probably the selection of different flavors arose. They started to select products with the most desired sensorial characteristics, using them as its essence to inoculate the subsequent batches of the product and to reproduce desired characteristics (Blandino, et al., 2003).

Cheese making for example, was the only way ancient people had to preserve the milk for long journeys. This art dates back 10,000 years ago in the Middle East, probably when milk stored in warm temperatures began to acidify due to the natural growth of lactic acid bacteria. This acidification generated different flavors and some of them where desirable in some products. Milk transportation in containers made with animal stomachs, where proteolytic enzymes and lactic acid bacteria are normally present, caused cheese formation and fermentation. Cheese also offers an additional advantage as it has higher nutrient concentrations than milk. The fermentation of milk in order to produce cheese, involves different stages as coagulation, acidification and water removal by the addition of salt (Stanley, 1998).

Another fermented product that has been preserved through history are fermented sausages. This is a traditional food that comes from south and central Europe, especially: Germany, Italy, Spain and France. Its first origins appear to be from the Roman Empire. This product is usually made of comminuted meat and fat, mixed with salt, sugar, spices and curing agents. All this ingredients are mixed and the sausage is stored for some time until the lactic acid bacteria completes the fermentation process (Lucke, 1998). The lactic acid produced by fermentation lowers the pH of the meat and becomes firm, it also prevent the growth of pathogenic bacteria. To increase the firmness it is necessary to let the sausage keep drying, this will also help to reduce water activity, which can help to prevent microbial growth and spoilage (Angel & Mora, 1992).

Fermented foods constitute a significant component in many diets around the world. In Africa for example, many fermented foods serve as main course meals and beverages. This kind of food is rich in raw carbohydrates. Some of the most representative include gari, ogi and mahewu wich are made from maize, and kaffir made from sorghum (Odunfa & Oyewole, 1998).

Fermented beverages have been produced since 1,700 BC. For example, the Greeks, Celts, Saxons and Vikings used honey to make wine. In countries like Egypt, Babylon, Rome and China the production of grape wine, and beer made from malted barley were common. In North America, the “pulque”, another similar fermented beverage made from agave was very popular. In South America, native communities produced a beer like beverage called “*chicha*”, made from grains and fruits. This beverage is used in rituals, gatherings, special occasions, business and work. It is consumed in high

volumes mainly by native people in the Amazon region (Alba-Lois & Segal-Kischinevzky, 2010).

Benefits of fermentation

There are various reasons why food may be fermented including preservation, inhibition of pathogens, improving nutritional value and organoleptic quality of the food, synthesis of flavoring, texturing compounds, psychotropic effects, etc (Bourdichon, et al., 2012). Fermentation also helps expanding the product shelf life through the formation of inhibitory metabolites such as organic acids, ethanol and bacteriocins. It also help the synthesis of some compounds as acetaldehyde, which gives the product special desired flavors. The texture of some products can be improved by the synthesis of extracellular polysaccharides, and enzymes that can produce protein hydrolysis of casein (Stanley, 1998). The conservation of fermented food is also increased by the inhibition of contaminant microorganisms. This is reached by the decrease of pH (due to the formation of lactic acid) to levels where most bacteria can´t survive. The decrease of pH by the use of lactic acid has been adopted in some industries to avoid the growth of contaminant bacteria and consequently the spoilage of the products (Steinkraus, 2006).

Fermented food provide nutritional and health benefits. In most of the cases, the nutritional content of fermented foods is higher than the one on its substrates. The fermentation process raise the protein content, and can improve the balance of essential amino acids. The availability of vitamins (thiamine, riboflavin, niacin and folic acid) content can be increased as well, having a direct effect on its consumers (Steinkraus, 2006). Natto for example, which is consumed in Japan for breakfast, has antioxidant

properties, and a high content of protein and vitamin K2, which may help to prevent osteoporosis. Tempe, an Indonesian fermented dish, has been claimed to reduce the cholesterol levels in blood; and like douchii (China), it has been reported that may lower the high blood pressure. Another fermented product is kimchi (Corea), there are reports that it can ameliorate osteoarthritis, liver disease, obesity and atherosclerosis. Gundruk (Himalayas), is a fermented vegetable product that has big concentrations of lactic and ascorbic acid, carotene and fibre. Pulque (Mexican fermented beverage extracted from cactus) has vitamins as thiamine, niacin, riboflavin, and biotin. These are just a few examples of all the range of fermented foods (and beverages) and their benefits.

There are four important fermentation processes: lactic acid, alcoholic, acetic acid and alkali fermentation. The one of our interest is lactic acid fermentation, and is mainly carried out by lactic acid bacteria. The most common fermenting bacteria are *Leuconostoc, Lactobacillus, Streptococcus, Pediococcus, Micrococcus* and *Bacillus*. This microbiome might change as time progresses in the fermentation process; this change may be due to differences in water activity, pH, salt content, temperature and composition of the substrate (Blandino, et al., 2003).

Traditional fermented foods are usually prepared from different cereals such as rice, wheat, sorghum, and corn. The microbiology of these products is not well known because of its complexity, and because it involves mixed cultures of bacteria and fungi. This mixture of microorganisms is variable during the different steps of fermentation. Some bacteria are predominant during the first stages of the fermentation process, but they decrease in time, so others become predominant (Blandino, et al., 2003).

Lactic acid bacteria (LAB)

Lactic acid bacteria is a heterogenous group of non-sporulating gram-positive organisms (rods or bacilli) characterized by a fermentative sugar metabolism, in which lactic acid is the major product (Herrero, et al., 1996). They can be anaerobic, microaerofilic or aerotolerant, also catalase and oxidase negative. They grow in an extended pH range that can oscillate from 3.2 to 9.6, but mostly they grow from 4 to 4.5 (Ulloa, et al., 2011).

This group of bacteria is widely distributed in nature, and they have been isolated from a wide spectrum of food products, soil, plants, digestive tract of mammals; among other sources. From the phylogenetic point of view there are 12 genera of lactic acid bacteria (Phylum: Firmicutes, Class: Bacilli, Order: Lactobacillales) *Lactobacillus, Lactococcus, Leuconostoc, Streptococcus, Pediococcus, Vagococcus, Enterococcus, Aerococcus, Tetragenococcus, Carnobacterium, Alloicoccus* and *Weisella* (Olivera, 2011).

The lactic acid bacteria has extended habitats including fruits and vegetables, soil, milk and milk products, nasopharyngeal and gastrointestinal tract. Many of these bacteria are present in humans and animals as part of the normal microbiome. For example infants, within the uterus are mostly sterile, but during birth they are exposed to different microorganisms, especially cocci that afterwards become normal microbiome that protect the skin and the mucosa from pathogens. When the baby is breast-fed, its intestinal tract become colonized by bacteria like *Bifidobacterium bifidus*, which can produce lactic acid, that give the baby protection to intestinal and respiratory diseases. In addition, some of the fermentative bacteria reside on plants and vegetables, which are eaten by animals including humans (Steinkraus, 2006).

The lactic acid bacteria can be of two types depending on the kind of final product obtained by its fermentation: homo or heterofermentative. Homofermentative bacteria (*Pediococcus, Streptococcus, Lactococcus* and some *Lactobacilli*) produce lactic acid as the major product from the glucose fermentation. Heterofermentative bacteria *(Weisella, Oenococcus, Carnobacterium, Lactosphaera, Leuconostoc* and some *Lactobacilli*), produce equivalent amounts of CO_2, ethanol and glucose, by the conversion of hexoses to pentoses (Blandino, et al., 2003).

Traditional fermented foods in Ecuador

In Ecuador fermented food and beverages has also been produced long time ago by different communities. Some of these products are cheese, champus, indigenous beer, sausages, vinegar, pulcre, chahuar mishque, nijimanch, puka, butter, guarapo, among others. This study was part of a bigger project where there was a collection of 63 artisanal fermented food samples including: cheese, champus (maize fermented beverage), chicha (maize, cassava or chonta beer), sausages, vinegar, pulcre (fermented juice extracted from *Agave americano*) and butter. These samples were obtained from inner communities from the three geographic regions (Pacific coast, Andean region and Amazon region) of the country. None of the products collected were made by starter cultures or lyophilized ferments (Cox, et al., 1987).

We analyzed 63 fermented food products from three geographical regions of Ecuador (Pacific coast, Andean region and Amazon region). All the products were made without any specific or commercial starter culture, and the process was completely artisanal. The samples included dairy products (fresh cheese, butter), meat products

(fermented sausages), sauces (vinegar), and alcoholic beverages (pulcre, champus, chonta beer, cassava beer, and maize beer). The consumption of these products is high in all the sites where they were collected, and its manufacture has been transmitted from generation to generation.

Chicha (grain based indigenous beer)

This is a fermented alcoholic beverage produced in countries as México, Guatemala, Colombia, Perú, Ecuador, Chile, and Bolivia (La Barre, 1938). The substrates used can be variable, and the main include corn (*Zea mays*), pineapple *(Ananas comosus*), chonta (fruit of *Bactris gasipaes*), peanut (*Arachis hypogaea*), wheat (*Triticum sativum*) and cassava (*Manihot esculenta*).

Archeological evidence in Huanuco Pampa (Perú) showed the presence of buildings believed to have housed women called *aqllas*. They were in charge of the preparation of this beer-like beverage for the state population especially for ceremonial practices (Hahn, 2009). Archeologists found thousands of jars or vessels, which indicates high production and consumption. This quantity of jars was also high because the weekly production had to be made in one batch, in order to have the time to ferment properly (Hayashida, 2008). In most communities, this traditional beer was an essential component of all of the meetings such as weddings, childbirths, funerals, or for work parties called “mingas” (La Barre, 1938). This beverage was also produced by families for daily consumption and by specialist brewers using maize collected from selected fields (Hayashida, 2008). The state also had to produce it for all the workers involved on the construction or maintenance of public roads and canals (Hahn, 2009).

The preparation of this beverage begins with the germination of the grain where the corn malt has to be soaked for 3 days at room temperature (20^{o}C). Then, the water is drained in order to let the grain germinate. When the germination is completed, an additional amount of water has to be added, so the mixture can be boiled for 30 minutes. The last step in this pre-fermenting stage consists on a filtration for the removal of the residues of grain (Vallejo, et al., 2013).

The vessel must be sealed for 24 hours, in order to reach the right conditions for fermentation. There are some ingredients that can be added as sugar cane, cinnamon or orange peel. The fermentation process can last from 15 to 20 days in beers made by cereals, and on the ones made by fruits and tubers this process lasts only 4 to 8 days (Lopez, et al., 2010).

There are many recipes for its production, all of them differ in little details characteristic from each community. In general, Andean brewers mill the grain before cooking it, and then they sieve the final product. In some cases, sediments are disposed, while in other communities they used it as food for people or animals (Hayashida, 2008).

The alcohol content can vary from 0.8 to 13.2%, but most of them have values around 5.8%. (Vallejo, et al., 2013). This level may increase by the addition of sweeteners as fruits or algarrobo pods. Another practice to improve this content was to let this beverage age for months or even years. This aged beer was consumed only in special occasions and it taste resemble to wine (Hayashida, 2008).

The container where fermentation takes place is generally made of ceramic with a very porous interior. The nature of this vessel let the fermenting bacteria to adhere to its

walls, and help the fermentation of following batches. Recently two strains of yeasts has been isolated from ancient vessels in tombs in Quito, Ecuador (Chang, et al., 2012).

There exists a kind of beer that has an unusual method of preparation, where the cassava or tuber from *Manihot esculenta* is chewed; leaving a starchy juice that will be fermented for the production of this alcoholic beverage. According to (Karsten, 1935) the saliva brought by the chewing helps the fermentation process (by providing amylases) and give the product a characteristic flavor and alcohol content (La Barre, 1938).

Saliva can serve as the source of amylase for the conversion of starch to fermentable sugars. The primary fermenting microorganisms are a mixture of bacteria and yeast including: *Saccharomyces cerevisiae, Lactobacillus sp., Leuconostoc sp., Acetobacter sp., and Aspergillus sp.* This traditional kind of beer is a big part of the South American culture, and it is known as a key element in social, political and ritual exchanges between some cultures (Blandino, et al., 2003).

PART II

SCIENTIFIC ARTICLE

Streptococcus salivarius, bacteria associated to human saliva is a major component of indigenous beer (*chicha*) from Ecuador

Ana L. Freire[1], Sonia Zapata[1], Juan Mosquera[1] y Gabriel Trueba[1]

[1] Microbiology Institute, Universidad San Francisco de Quito, Quito, Ecuador.

ABSTRACT

Indigenous beers (chicha) play an important role in cultural events of indigenous people from Ecuador. In general, all the Ecuadorian production of indigenous beer is artisanal and it does not include the addition of any starter cultures or lyophilized ferments. The fermentation depends mostly on microorganisms from the environment (including vessels from previous batches of fermented beverage). We analyzed the microbiota of artisanal beers using bacterial cultures and 16S-based tag-encoded FLX amplicon pyrosequencing (bTEFAP). As previous reports we found that *Lactobacillus* sp. is predominantly present in most types of Ecuadorian chicha; however we found that *Streptococcus salivarius* and S*treptococcus mutans* (part of the human mouth microbiota)

were also part of microbiota of chewed beers from the Amazon region. We also demonstrated that *S. salivarius* and *S. mutans* could proliferate in cassava mush.

Subjects: Microbiology, Food processing, Molecular Biology

Key words: indigenous beer, *Streptococcus salivarius*, artisanal fermented beverages, *Streptococcus mutans*, fermented cassava, lactic acid bacteria, saliva, chewed indigenous beer.

INTRODUCTION

The domestication of fermenting bacteria and yeast may have predated the domestication of animals and plants, as ancestral hominids adapted to metabolize alcohol long time before the Neolithic (Carrigan, et al., 2015). The organoleptic and psychotropic effects associated to the consumption of fermented fruits may have motivated early humans to replicate the process. Fermentation may have provided unintended benefits as fermenting bacteria may have protected ancient societies from dangerous food borne pathogens such as *Salmonella* or *Listeria* (Nakamura, et al., 2012) (Lewus, et al., 1991) (Fooks & Gibson, 2002) (Tesfaye, et al., 2011) (Agapakis & Tolaas, 2012). 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 of these fermenting microorganisms (Libkinda, et al., 2011).

Many fermenting microorganisms have originated in the environment (Martini, 1993), others resemble those found in the human microbiome (Agapakis & Tolaas, 2012) suggesting that some microorganisms used for food fermentation may have originated

from human microbiome (skin and intestine); modern fermented dairy products contain intestinal bacteria (Jens, 2008).

Indigenous people from South America (such as Ecuador) prepare a type of beer known as chicha which is prepared with corn, cassava or a fruit of the palm *Bactris gasipaes* (chonta). In Ecuador, some of these beers are prepared by chewing boiled cassava and spiting into a container. This type of beer is consumed as part of rituals in some cultures of the Amazon region. Microorganisms from the environment (including those from containers) and enzymes from saliva (in the case of chewed beers) are thought to contribute in the fermentation process. We analyzed the microbial diversity in beer from corn, chonta, chewed and mushed cassava.

MATERIALS AND METHODS

Sample collection

Four samples of *chicha* (indigenous beer) from two geographical regions of Ecuador (Andean and Amazon region) were collected. This samples included beer made of: chewed cassava, mushed cassava, chonta and corn (Table 1). All these products were obtained from rural communities, which elaborated them in artisanal way. None of them were pasteurized, or had any commercial additives or preservatives. After collection, all the samples were refrigerated (2 to 8^{o} C). Additionally, the same day of collection, a 2 mL aliquot of sample was stored at -20^{o}C, for microbial 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), ten-fold dilutions were made up to 10^{-6} 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°C and 43°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 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 or bacilli) were conserved for further characterization. From each colony the 16s gene was amplified and sequenced. Strains were stored at -20°C in MRS or M17 broth with 20% of glycerol.

Genotypic characterization

DNA extraction was performed with the DNAzol Reagent (Life Technologies, 2001). One pure 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 (NCBI).

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. mutants* contamination, one colony of a pure culture of each bacteria was diluted in 25mL of sodium citrate (2%) separately. Subsequently, 1 mL of this cell suspension was used to inoculate tubes containing 9mL of chewed cassava solution (10%) and incubated at 37^{o}C under anaerobiosis. 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 after the inoculation. Results from each day were compared to determine the ability of these bacteria to grow in chewed cassava solution.

Statistical analysis

All counts were analyzed by the Mann-Whitney U test to determine if there was significant difference from one day to the next one.

RESULTS

Characterization of bacterial isolates obtained by traditional culture

Twenty-five bacterial isolates were characterized by 16s rDNA sequencing (Table 2). The most predominant species with a 16% were *Lactobacillus fermentum, Lactococcus lactis*, *Leuconostoc mesenteroides* and *Streptococcus salivarius*; followed by *Lactobacillus*

plantarum and Weissella confusa both in an 8%. Finally, with a 4% of abundance there were *Lactobacillus casei, Lactobacillus pantheris, Lactobacillus parabuchneri, Lactobacillus paracasei* and *Streptococcus mutans*. These results had 99% to 100% identity percentage when compared to the GenBank Database.

High throughput sequencing analysis

The pyrosequencing analysis of the four beer samples showed the presence of 140 different species belonging to 49 genera of bacteria. The most predominant genus are *Lactobacillus* (28.8%), closely followed by *Streptococcus* (26.4%). However, at species level, the most abundant was *Streptococcus salivarius* (24.2%) as it is exposed in Table 3. The corn beer (CoB) showed the highest genus diversity (29 genera), followed by the chewed cassava beer (CC) and the chonta beer (CB) with 20 genera each. The less diverse was the mushed cassava beer (MC) showing just 13 genus detected. In Table 3 are exposed the 20 most abundant species of each type of beer.

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

S. salivarius (Figure 1) and S. mutans (Figure 2) grow in chewed cassava solution. These results were significative as shown by corroborated by Mann-Whitney U test for both microorganisms. The difference in the growth time in the cassava starch solution corresponds to the time needed during both strains isolation in MRS and M17 media. After 48 hours (*S. salivarius*) and 72 hours (*S. mutans*) of culture, the bacteria began to die.

DISCUSION

This study shows that *S. salivarius* and *S. mutans* are present in large numbers not only in the chewed cassava beers but in the non-chewed beers as well. Both of these bacteria are part of the of the oral cavity microbiome and potential pathogens. The presence of these microorganisms in beer without human saliva indicates that *S. salivarius* and *S. mutans* can proliferate as confirmed by our experiments (Figure 1 and 2). Therefore, saliva not only plays an important role speeding up the fermentation process (by providing amylases) (Henkel, 2005), but also provides fermenting bacteria such as *S. salivarius* and *S. mutans*. *Streptococcus salivarius* is a homofermenter and is closely related to *Streptococcus thermophilus,* which is one of the microorganisms mostly used as starter cultures (Burton, et al., 2006); *S. mutans* is one of the principal causative agents of dental plaque and dental cavities (Loesche, 1986) ; (Corby, et al., 2005). *Streptococcus mutans* can be transmitted from person to person, and it is more prevalent in adults than in children (Fischetti, et al., 2006) ; (Corby, et al., 2005).

One recent study (Colehour, et al., 2014) failed to detect *S. mutans* and *S. salivarius* in beer samples. This may be due to several reasons as the site of collection, fermentation time, bacteria behavior (autolysis), presence of other interfering bacteria, among others. The duration of the fermentation process may be a crucial parameter in the isolation of viable bacteria, as the microbiota present might change every day. For example at day three (young beer) the predominant microbiota is more diverse than the one observed in a mature beer (six or more days). Additionally in a young beer, the predominant microbiota might be the one characteristic of the main ingredient (corn, cassava, chonta), and not the microbiota of the fermented product (Steinkraus, 2006).

The beers analyzed by Colehour et al 2014 were mature (more than 4 days); this differed from the present study; our samples were collected and stored (at -20^{o}C) at day 3 stopping the fermentation process and maintaining the initial characteristic microbiota.

The absence of these streptococci in previous reports may be the result of physiological characteristics of these bacteria. The growth of *S. salivarius* and *S. mutans* in chewed cassava solution was followed by a sudden drop in colony numbers after 24 (*S. salivarius*) and 48 hours (*S. mutans*) of culture. This may be due to the consumption of all the nutrients in the cassava solution or autolysis. The latter phenomenon is induced by quorum sensing in response to stress (Dufour & Lévesque, 2013). Also, these bacteria are known to form biofilm (Ajdic, et al., 2002) in response to quorum sensing (Li, et al., 2002) which changes the bacterial behavior from planktonic to sessile and causing the reduction of bacterial counts after 24h. Unfortunately, we did not attempt to recover sessile bacteria in this study. Additionally, Colehour, et al., 2014 found predominance of *L. reuteri* which is known to antagonize *S. salivairus* (Nikawa, et al., 2004) ; (Corby, et al., 2005); this bacterium was not found in our study which may explain the discrepancy, in the presence of salivary bacteria, of both studies. Biofims of *S. mutans* and *S. salivarius* might antagonize with *Streptococcus sanguinis* (Ajdic, et al., 2002), a species also found in our study (Table 3).

Similar to (Colehour, et al., 2014) investigation, *Lactobacillus* was the dominant genus by bacteriological culture and high throughput analysis in our study. Furthermore, compared to our study, the work of (Elizaquivel, et al., 2010) and (Puerari, et al., 2015) also showed similar results of genera found in indigenous beer (corn and rice). Next

generation sequencing analysis of corn beer by Balordi (2015) obtained *Lactobacillus* as the most predominant genus, whereas by traditional culture *Enterococcus* was the most abundant (Balordi, 2015).

High throughput sequencing allowed us to identify microorganisms, which were not detectable by bacterial culture: *L. brevis, S. parasanguinis, S. pasteurianus, L. camelliae, L. fermentum, L. paracasei, S. pneumoniae, S. oralis, L. parabuchneri, S. vestibularis, S. cristatus, W. confusa, L. paracollinoides, L. manihotivorans, L. vaccinostercus, C. maltaromaticum, L. pentosus, E. cancerogenus, B. amyloliquefaciens, E. asburiae, among others*. Some of them are opportunistic pathogens: *S. pnemunoniae, E. faecium, S. gallolyticus, S. pseudopneumoniae* and *S. salivarius* (Fisher & Phillips, 2009); (Steinkraus, 2006).

CONCLUSIONS

By the use of classical and molecular approaches, it was possible to analyze the microbiome of indigenous beers. It was surprising to find *Streptococcus salivarius* and *Streptococcus mutans*, bacteria associated to human saliva were two bacterial components of this beverage. The presence of both species was probably not due to bacterial (transitory presence) or DNA contamination because they were able to grow in a chewed cassava solution. The presence of *S. salivarius* in non-chewed cassava beer may indicate that this bacterium has the aptitude to grow in chewed cassava solutions and probably survive in the fermenting containers.

Finally, this study suggests that variation in the beer microbiota composition may depend on geographical location, ingredients, and duration of the fermentation process.

BIBLIOGRAPHY

Agapakis, C., & Tolaas, S. (2012). Smelling in multiple dimensions. Current opinion in chemical biology. *16*(5), 569-575.

Ajdic, D., McShan, W., McLaughlin, R., Savic, G., Chang, J., Carson, M., & Ferreti, J. (2002). Genome sequence of Streptococcus mutans UA159, a cariogenic dental pathogen. *PNAS, 99*(22), 14434-14439.

Alba-Lois, L., & Segal-Kischinevzky, C. (2010). Yeast Fermentation and the Making of Beer and Wine. *Nature Education, 3*(9), 17.

Angel, S., & Mora, E. (1992). *Applications of Biotechnology to Traditional Fermented Foods: Fermented Sausages.* Washington D.C. : National Academy Press.

Balordi, M. (2015). *Microbiota of Andean Food: tradition for healthy products.* Milán: CORDIS.

Blandino, Al-Aseeri, Pandiella, Cantero, & Webb. (2003). Cereal-based fermented foods and beverages. *Food Research International*, 527-543.

Bourdichon, Casaregola, Farrokh, Frisvad, Gerds, Hammes, . . . Seto. (2012). Food Fermentations: Microorganisms with technological beneficial use. *International Journal of Food Microbiology*(154), 87-97.

Burton, J., Wescombe, P., Moore, C., Chilcott, C., & Tagg, J. (2006). Safety Assessment of the Oral Cavity Probiotic Streptococcus salivarius K12. *Applied Environmental Microbiology, 72*(4), 3050-3053.

Carrigan, M., Uryasev, O., Frye, C., Eckman, B., Myers, C., Hurley, T., & Benner, S. (2015). Hominids adapted to metabolize ethanol long before human-directed fermentation. *PNAS, 112*(2), 458–463.

Chang, Lin, Chen, Carvaja, Portero, James, . . . Lee. (2012). Candida theae sp. nov., a new anamorphic beverage-associated member of the Lodderomyces clade. *International Journal of Food Microbiology, 153*(1), 10-14.

Colehour, A., Meadow, J., Liebert, M., Cepon-Robins, T., Gildner, T., Urlacher, S., . . . Sugiyama, L. (2014). Local domestication of lactic acid bacteria via cassava beer fermentation. *Peer Journal*, 479.

Corby, Lyons-Weiler, Bretz, Hart, Aas, Boumenna, . . . Paster. (2005). Microbial Risk Indicators of Early Childhood Caries. *Journal of Clinical Microbiology, 43*(11), 5753-5759.

Cox, L., Caicedo, B., Vanos, V., Heck, E., Hofstaetter, S., & Cordier, L. (1987). A catalogue of some Ecuadorean fermented beverages, with notes on their microflora. *MIRCEN Journal, 3*, 143-153.

Dufour, D., & Lévesque, C. (2013). Cell Death of Streptococcus mutans Induced by a Quorum-Sensing Peptide Occurs via a Conserved Streptococcal Autolysin. *Journal of Bacteriology, 195*(1), 105-114.

Elizaquivel, P., Aristimuño, C., Cocconcelli, P., Vignolo, G., & Azar, R. (2010). Molecular identification of lactic acid bacteria associated with Chicha, a traditional maize-based fermented beverage from Northwestern Argentina. *Centro de Referencia para Lactobacillus*.

Fischetti, V., Novick, R., Ferretti, J., Portnoy, D., & Rood, J. (2006). *Gram-Positive Pathogens* (2nd ed.). Washington: American Society for Microbiology press.

Fisher, K., & Phillips, C. (2009). The ecology, epidemiology and virulence of Enterococcus. *Microbiology, 155*(6), 1749-1757.

Fooks, L., & Gibson, G. (2002). In vitro investigations of the effect of probiotics and prebiotics on selected human intestinal pathogens. *FEMS Microbiology Ecology, 39*(1), 67-75.

Hahn, R. (2009). Andean commensal politics and alternative rituals of power at Jatanca, Peru. *McGill University, 1*(1), 1-129.

Hayashida, F. (2008). Ancient beer and modern brewers: Ethnoarchaeological observations of chicha production in two regions of the North Coast of Peru. *Journal of Anthropological Archaeology, 27*, 161-174.

Henkel, T. (2005). Parakari, an indigenous fermented beverage using amylolytic Rhizopus in Guyana. *The Mycological Society of America, 97*(1), 1-11.

Herrero, Mayo, Gonzalez, & Suárez. (1996). Evaluation of technologically important traits in lactic acid bacteria isolated from spontaneous fermentations. *Journal of Applied Bacteriology*(81), 565-570.

Jens, W. (2008). Ecological role of lactobacilli in the gastrointestinal tract: implications for fundamental and biomedical research. *Applied and environmental microbiology, 74*(16), 4985-4996.

Joffe, A. (1998). Alcohol and complexity in ancient western Asia. *The University of Chicago Press, 39*(3), 297-322.

Karsten, R. (1935). *The Head-Hunters of Western Amazons: The Life and Culture of the Fibaro Indians of Eastern Ecuador and Peru.* Helsinki: Societas Scientiarum.

La Barre, W. (1938). *Native American Beers* (1 ed.). New York: Amer Anthropologist.

Lewus, C., Kaiser, A., & Montville, T. (1991). Inhibition of food-borne bacterial pathogens by bacteriocins from lactic acid bacteria isolated from meat. *Applied and Environmental Microbiology, 57*(6), 1683-1688.

Li, Tang, Aspiras, Lau, Lee, Ellen, & Cvitkovitch. (2002). A quorum-sensing signaling system essential for genetic competence in Streptococcus mutans is involved in biofilm formation. *Journal of Bacteriology, 184*(10), 2699-2708.

Libkinda, D., Hittingerb, C., Valériod, E., Gonçalvesd, C., Doverb, J., Johnston, M., . . . Sampaio, J. (2011). Microbe domestication and the identification of the wild genetic stock of lager-brewing yeast. *Proceedings of the National Academy of Sciences, 108*(35), 14539–14544.

Life Technologies. (2001). DNAzol reagent Protocol. Thermo Fisher Scientific Inc.

Loesche, W. (1986). Role of Streptococcus mutans in Human Dental Decay. *Microbiological Reviews, 50*(4), 353-380.

Lopez, W., Ramirez, M., Mambuscay, L., & Osorio, E. (2010). Yeast diversity associated to Colombian traditional "chichas". *Revista Colombiana de Biotecnología, 12*(2), 176-186.

Lucke. (1998). *Microbiology of fermented foods: Fermented Sausages.* Thomson Science.

Martin, F., Philippot, L., Hallet, S., Chaussod, R., Germon, J., Soulas, G., & Catroux, G. (2001). DNA extraction from soils: old bias for new microbial diversity analysis methods. *Applied and Environmental Microbiology, 67*, 2354-2359.

Martini, A. (1993). Origin and domestication of the wine yeast Saccharomyces cerevisiae. *Journal of Wine*, 165-176.

Nakamura, S., Kuda, T., An, C., Kanno, T., Takahashi, H., & Kimura, B. (2012). Inhibitory effects of Leuconostoc mesenteroides 1RM3 isolated from narezushi, a fermented fish with rice, on Listeria monocytogenes infection to Caco-2 cells and A/J mice. *Anaerobe, 18*(1), 19-24.

Nikawa, Makihira, Fukushima, Nishimura, Ozaki, Ishida, . . . Aimi. (2004). Lactobacillus reuteri in bovine milk fermented decreases the oral carriage of mutans streptococci. *International Journal of Food Microbiology, 95*(2), 219-223.

Odunfa, & Oyewole. (1998). *Microbiology of Fermented Foods: African fermented foods.* Thomson Science.

Olivera, J. (2011). Caracterización tecnológica de cepas de bacterias ácido lácticas aisladas de la leche. *Unidad Tecnológica de Alimentos - Facultad de Agronomía*, 1-45.

Puerari, C., Teixeira, K., & Freitas, R. (2015). Physicochemical and microbiological characterization of chicha, a rice-based fermented beverage produced by Umutina Brazilian Amerindians. *Food Microbiology, 46*, 210-217.

Stanley. (1998). *Microbiology of Fermented Foods: Cheeses.* Thomson Science.

Steinkraus. (2006). Fermentations in World Food Processing. *Comprehensive Reviews in Food Science and Food Safety, 1*, 23-32.

Tesfaye, A., Mehari, T., & Ashenafi, M. (2011). Inhibition of some foodborne pathogens by pure and mixed LAB cultures during fermentation and storage of Ergo, A traditional Ethiopian fermented milk. *ARPN Journal of Agricultural & Biological Science, 6*(4), 13-19.

Ulloa, Ramirez, Velásquez, & Arce. (2011). Lactic Acid Bacteria: Importance in food products and their effects in human health. *Revista Fuente, 7*.

Vallejo, Miranda, Flores, Sanchez, Ageitos, Gonzales, . . . Villa. (2013). Atypical yeasts identified as Saccharomyces cerevisiae by MALDI-TOFMS and gene sequencing are the main responsible of fermentation ofchicha, a traditional beverage from Peru. *Systematic and Applied Microbiology*(36), 560-564.

FIGURES

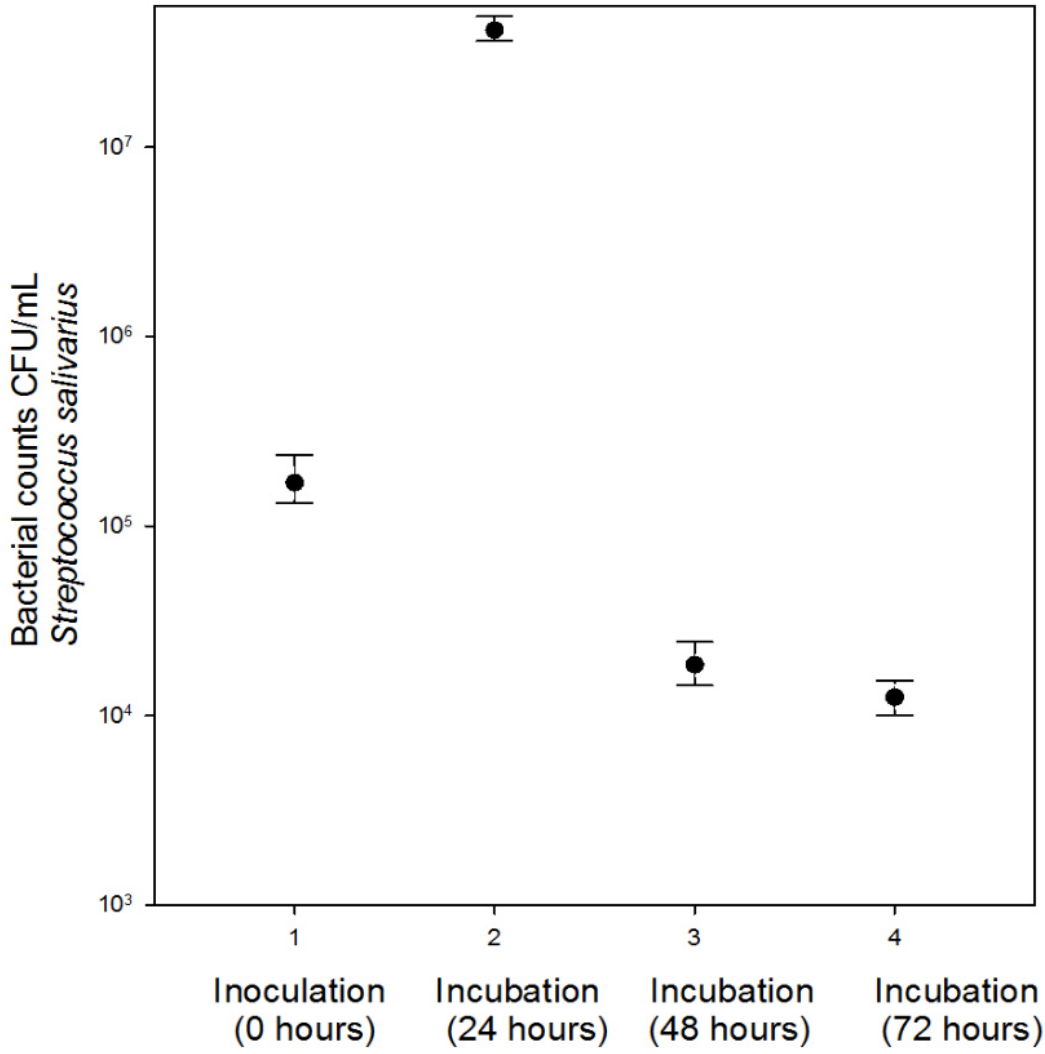

Figure 1 Growth of *S. salivarius* in chewed cassava solution. There is a significate increase in CFU at the 24 hours of incubation compared to the inoculation time (0 hours). During the second day of incubation (48 hours), the bacteria began to die due to the lack of nutrients.

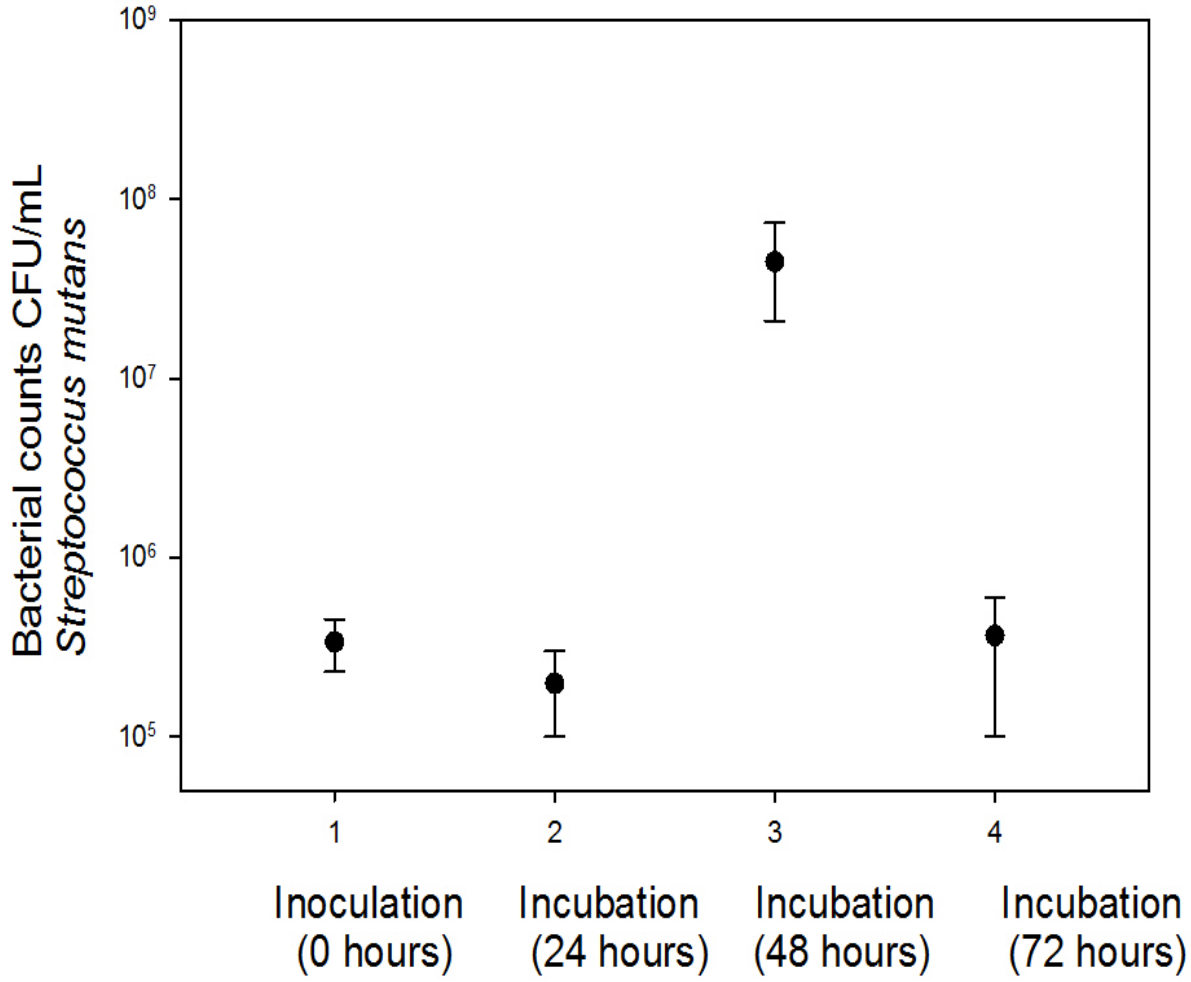

Figure 2 Growth of *S. mutans* in chewed cassava solution. There is a significate increase in CFU at the 48 hour of incubation compared to the inoculation time (0 hours). During the third (72 hours) day of incubation, the bacteria began to die due to the lack of nutrients.

TABLES

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

Main ingredient	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

Table *2.* Bacteria isolated by traditional culture from the four beer samples. All the 25 strains were obtained by MRS and M17 culture, and subsequently amplified (16s ribosomal gene) by PCR. *Leuconostoc mesenteroides, Lactobacillus fermentum, Lactococcus lactis* and *Streptococcus salivarius* had a 16% of abundance, followed by *Lactobacillus plantarum* and *Weissella confusa* (8%). Finally with a 4% were *Streptococcus mutans, Lactobacillus casei, Lactobacillus paracasei, Lactobacilus parabuchneri* and *Lactobacillus pantheris.* Bacterial identities were acquired by a BLAST search on the NCBI database.

Sample	Isolate ID	Culture Media	Growth condition	Identification (16S)
Chewed	25 A2	MRS	Anaerobic	*Leuconostoc mesenteroides*
cassava	25 C2	MRS	Aerobic	*Lactobacillus fermentum*
beer	25 E2	M17	Anaerobic	*Streptococcus mutans*
	25 F1	M17	Aerobic	*Lactococcus lactis*
	25H1	M17	Aerobic	*Streptococcus salivarius*
Mushed	26 A1	MRS	Anaerobic	*Lactobacillus fermentum*
cassava	26 B1	MRS	Anaerobic	*Lactobacillus fermentum*
beer	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	27 A1	MRS	Anaerobic	*Lactobacillus plantarum*
beer	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*

Table 3. Most predominant species found by pyrosequencing analysis in the four beer samples (abundance of more than 0.1%). These data is based on the comparison of the four beer samples high throughput sequencing (CD Genomics). Chewed cassava beer (CC), mushed cassava beer (MC), chonta beer (CB) and corn beer (CoB). The total percentage value it is the sum of the percentages obtained from the four samples.

Species	Percentage of abundance per Sample				Detected in culture	Possible origin
	CC	MC	CB	CoB		
Streptococcus salivarius	31.94	65.05	0.00	0.00	Yes	oral microflora
Weissella confusa	0.47	0.06	45.92	25.33	Yes	vegetables
Weissella sp	0.20	0.34	19.80	19.36	No	vegetables
Lactobacillus plantarum	10.86	0.00	12.40	0.12	Yes	gut
Lactobacillus paracollinoides	0.00	0.00	0.00	15.98	No	beer spoilage
Lactobacillus brevis	8.39	0.12	2.50	0.61	No	gut
Lactococcus lactis	2.10	0.03	8.91	0.01	Yes	environment
Lactococcus sp	0.17	9.32	0.99	0.21	No	gut
Lactobacillus fermentum	6.50	3.78	0.03	0.00	Yes	gut
Streptococcus parasanguinis	5.41	3.47	0.00	0.00	No	oral microflora
Lactobacillus paracasei	0.00	0.00	0.00	8.63	Yes	gut, food
Lactobacillus delbrueckii	8.05	0.00	0.00	0.00	No	gut, vegetables
Streptococcus pasteurianus	0.00	7.74	0.00	0.00	No	gut
Lactobacillus camelliae	0.03	0.00	0.00	7.29	No	gut
Lactobacillus sp	3.41	0.00	0.72	1.26	No	environment
Streptococcus sp	2.54	2.29	0.07	0.04	No	gut
Leuconostoc sp	0.00	0.03	0.07	4.59	No	vegetables
Streptococcus pneumoniae	3.65	0.46	0.00	0.00	No	nasopharynx
Streptococcus thermophilus	1.25	2.59	0.00	0.00	No	vegetables
Fructobacillus sp	0.00	0.00	0.00	3.84	No	vegetables
Lactobacillus casei	0.00	0.00	0.00	3.13	Yes	gut
Lactococcus garviae	0.00	0.00	2.76	0.00	No	fermented food
Leuconostoc citreum	0.00	1.52	1.22	0.01	No	fermented food
Leuconostoc lactis	1.69	0.06	0.16	0.82	No	environment
Lactobacillus harbinensis	0.00	0.00	0.00	2.07	No	vegetables
Weissella cibaria	0.07	0.03	0.92	0.93	No	vegetables
Lactobacillus manihotivorans	1.83	0.00	0.00	0.02	No	vegetables
Streptococcus oralis	1.39	0.24	0.00	0.00	No	oral microflora
Lactobacillus vaccinostercus	1.22	0.00	0.23	0.00	No	environment
Lactobacillus parabuchneri	0.00	0.00	0.00	1.40	Yes	oral microflora
Enterobacter sp	1.32	0.00	0.07	0.00	No	gut
Oenococcus kitaharae	0.00	0.00	0.00	1.20	No	vegetables
Streptococcus vestibularis	0.37	0.79	0.00	0.00	No	oral microflora
Serratia sp	0.98	0.00	0.03	0.02	No	environment
Carnobacterium maltaromaticum	0.00	0.00	0.95	0.06	No	environment
Weissella paramesenteroides	0.47	0.00	0.10	0.00	No	environment
Streptococcus gallolyticus	0.00	0.55	0.00	0.00	No	tumors

Gluconacetobacter intermedius	0.00	0.00	0.00	0.55	No	fermented food
Streptococcus pseudopneumoniae	0.54	0.00	0.00	0.00	No	pathogen
Lactobacillus pentosus	0.24	0.00	0.30	0.01	No	environment
Enterobacter cancerogenus	0.47	0.00	0.03	0.00	No	environment
Bacillus amyloliquefaciens	0.00	0.49	0.00	0.00	No	environment
Enterobacter asburiae	0.47	0.00	0.00	0.00	No	environment
Streptococcus cristatus	0.41	0.00	0.00	0.00	No	oral microflora
Lactobacillus guizhouensis	0.37	0.00	0.00	0.02	No	vegetables
Kluyvera ascorbata	0.37	0.00	0.00	0.00	No	gut, food

Table 4. Comparison of the 20 most predominant species in the 4 beer samples analyzed by pyrosequencing.

Chewed cassava beer		Mushed cassava beer		Chonta beer		Corn beer	
Species	**%**	***Species***	**%**	***Species***	**%**	***Species***	**%**
Streptococcus salivarius	31.94	*Streptococcus salivarius*	65.05	*Weissella confusa*	45.92	*Weissella confusa*	25.33
Lactobacillus plantarum	10.86	*Lactococcus sp*	9.32	*Weissella sp*	19.80	*Weissella sp*	19.36
Lactobacillus brevis	8.39	*Streptococcus pasteurianus*	7.74	*Lactobacillus plantarum*	12.40	*Lactobacillus paracollinoides*	15.98
Lactobacillus delbrueckii	8.05	*Lactobacillus fermentum*	3.78	*Lactococcus lactis*	8.91	*Lactobacillus paracasei*	8.63
Lactobacillus fermentum	6.50	*Streptococcus parasanguinis*	3.47	*Lactococcus garviae*	2.76	*Lactobacillus camelliae*	7.29
Streptococcus parasanguinis	5.41	*Streptococcus thermophilus*	2.59	*Lactobacillus brevis*	2.50	*Leuconostoc sp*	4.59
Streptococcus pneumoniae	3.65	*Streptococcus sp*	2.29	*Leuconostoc citreum*	1.22	*Fructobacillus sp*	3.84
Lactobacillus sp	3.41	*Leuconostoc citreum*	1.52	*Lactococcus sp*	0.99	*Lactobacillus casei*	3.13
Streptococcus sp	2.54	*Streptococcus vestibularis*	0.79	*Carnobacterium maltaromaticum*	0.95	*Lactobacillus harbinensis*	2.07
Lactococcus lactis	2.10	*Streptococcus gallolyticus*	0.55	*Weissella cibaria*	0.92	*Lactobacillus parabuchneri*	1.40
Lactobacillus manihotivorans	1.83	*Bacillus amyloliquefaciens*	0.49	*Lactobacillus sp*	0.72	*Lactobacillus sp*	1.26
Leuconostoc lactis	1.69	*Streptococcus pneumoniae*	0.46	*Lactobacillus pentosus*	0.30	*Oenococcus kitaharae*	1.20
Streptococcus oralis	1.39	*Weissella sp*	0.34	*Lactobacillus vaccinostercus*	0.23	*Weissella cibaria*	0.93
Enterobacter sp	1.32	*Streptococcus oralis*	0.24	*Leuconostoc lactis*	0.16	*Leuconostoc lactis*	0.82
Streptococcus thermophilus	1.25	*Lactobacillus brevis*	0.12	*Acetobacter orientalis*	0.16	*Lactobacillus brevis*	0.61
Lactobacillus vaccinostercus	1.22	*Streptococcus peroris*	0.12	*Aquimarina sp*	0.16	*Gluconacetobacter intermedius*	0.55
Serratia sp	0.98	*Klebsiella sp*	0.12	*Enterococcus sp*	0.13	*Acetobacter sp*	0.26
Streptococcus pseudopneumoniae	0.54	*Leuconostoc kimchii*	0.09	*Lactobacillus nantensis*	0.13	*Gluconobacter oxydans*	0.22
Enterobacter asburiae	0.47	*Leuconostoc lactis*	0.06	*Lactobacillus kimchii*	0.13	*Lactococcus sp*	0.21
Enterobacter cancerogenus	0.47	*Weissella confusa*	0.06	*Weissella paramesenteroides*	0.10	*Lactobacillus zeae*	0.21
Others	6.06	*Others*	0.79	*Others*	1.39	*Others*	2.12

APENDIX

LACTIC ACID BACTERIA ISOLATED FROM ARTISANAL FERMENTED PRODUCTS OF ECUADOR

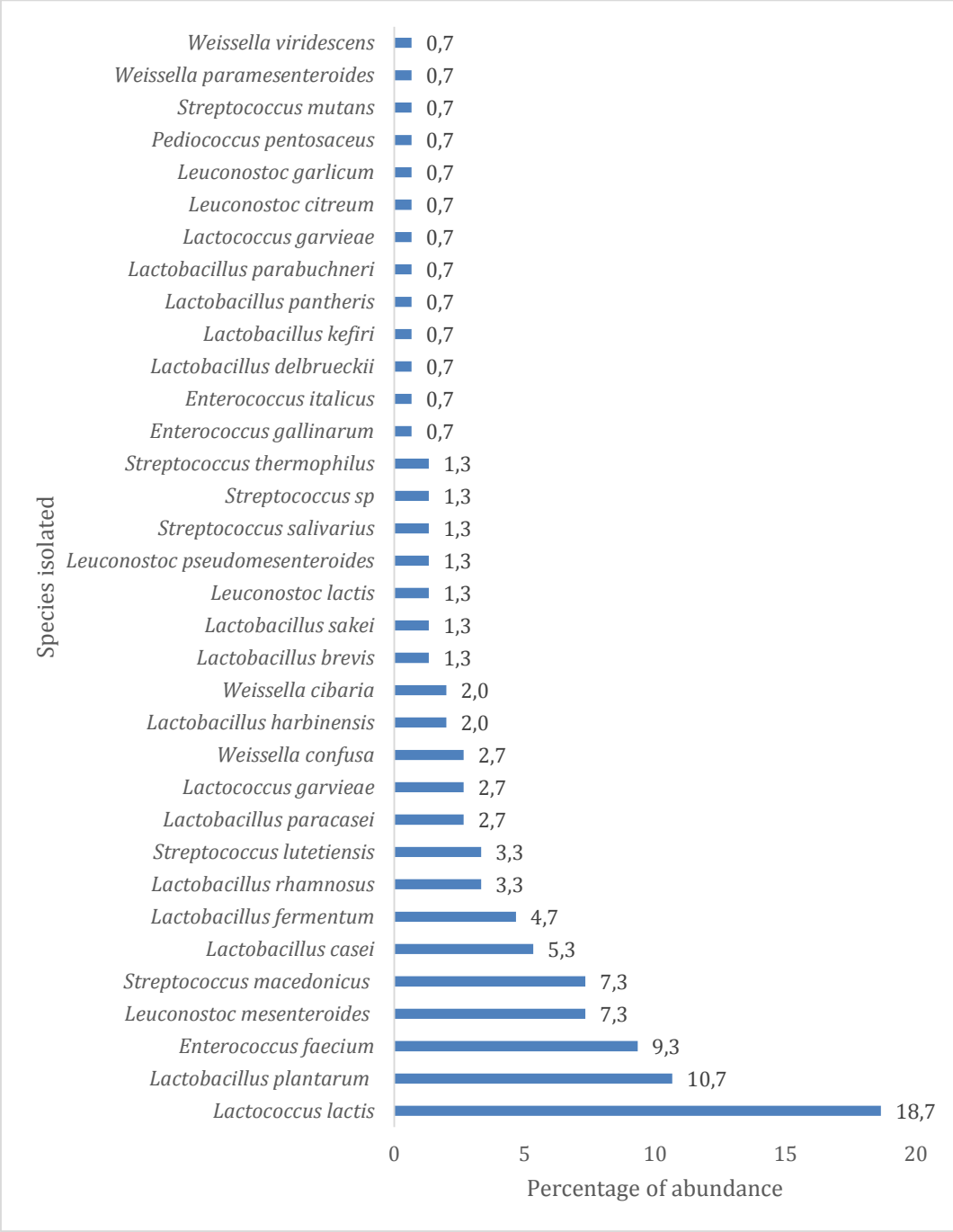

Figure 1. Percentage of abundance of lactic acid bacteria isolated from the 63 artisanal fermented food products collected in Ecuador (Pacific coast, Andean region and Amazon region). The identities of the strains were acquired by sequencing of the 16s ribosomal gene, followed by a blast search in the NCBI GenBank Database of the isolates obtained by culture in MRS and M17 culture media of the fermented products.

Table 1. Microorganisms isolated from the eight beer samples and identified by the sequencing of the 16S gene. The identities of the strains were acquired by sequencing of the 16s ribosomal gene, followed by a blast search in the NCBI GenBank Database of the isolates obtained by culture in MRS and M17 culture media of the fermented products.

Sample	Isolate ID	Culture Media	Growth condition	Identification (16S)
Chewed	25 A2	MRS	Anaerobic	*Leuconostoc mesenteroides*
cassava	25 C2	MRS	Aerobic	*Lactobacillus fermentum*
beer	25 E2	M17	Anaerobic	*Streptococcus mutans*
	25 F1	M17	Aerobic	*Lactococcus lactis*
	25H1	M17	Aerobic	*Streptococcus salivarius*
Mushed	26 A1	MRS	Anaerobic	*Lactobacillus fermentum*
cassava	26 B1	MRS	Anaerobic	*Lactobacillus fermentum*
beer	26 C2	MRS	Aerobic	*Lactobacillus fermentum*
	26 E2	M17	Anaerobic	*Streptococcus salivarius*
	26 F2	M17	Anaerobic	*Streptococcus sp*
	26 G1	M17	Aerobic	*Streptococcus sp*
Chonta	27 A1	MRS	Anaerobic	*Lactobacillus plantarum*
beer	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	58 A1	MRS	Anaerobic	*Lactobacillus harbinensis*
	58 A2	MRS	Anaerobic	*Lactobacillus harbinensis*
	58 B1	MRS	Aerobic	*Lactobacillus fermentum*
	58 B2	MRS	Aerobic	*Lactobacillus harbinensis*
Corn beer	59 C1	M17	Anaerobic	*Streptococcus macedonicus*
(with sugar)	59 C2	M17	Anaerobic	*Streptococcus macedonicus*
	59 D1	M17	Anaerobic	*Streptococcus macedonicus*
	59 D2	M17	Anaerobic	*Streptococcus macedonicus*
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*
Corn beer	62 A1	MRS	Anaerobic	*Lactobacillus plantarum*
	62 A2	MRS	Anaerobic	*Lactobacillus plantarum*
	62 B1	MRS	Aerobic	*Lactobacillus casei*
	62 C1	MRS	Aerobic	*Lactobacillus plantarum*
	62 D1	M17	Anaerobic	*Lactobacillus paracasei*
	62 E1	M17	Anaerobic	*Lactobacillus casei*
	62 F1	M17	Aerobic	*Lactobacillus paracasei*
	62 F2	M17	Aerobic	*Lactobacillus casei*
Corn beer	63 A1	MRS	Anaerobic	*Weissella confusa*
	63 A2	MRS	Anaerobic	*Weissella cibaria*
	63 B1	MRS	Anaerobic	*Lactobacillus plantarum*
	63 C2	MRS	Aerobic	*Weissella cibaria*
	63 D2	MRS	Aerobic	*Lactococcus lactis*
	63 E1	M17	Anaerobic	*Lactococcus lactis*
	63 F1	M17	Aerobic	*Leuconostoc mesenteroides*

Table 2. Microorganisms isolated from cheese samples and identified by the sequencing of the 16S gene. The identities of the strains were acquired by sequencing of the 16s ribosomal gene, followed by a blast search in the NCBI GenBank Database of the isolates obtained by culture in MRS and M17 culture media of the fermented products.

Type of cheese	Sample number	Isolate	Culture media	Culture conditions	Region	Identification
Cheese (natural rennet)	46	A2	MRS	Anaerobic	Andean	*Lactobacillus brevis*
	47	A2	MRS	Anaerobic	Andean	*Leuconostoc lactis*
	47	D1	M17	Aerobic	Andean	*Lactococcus lactis*
Cheese with salt	20	C1	MRS	Aerobic	Amazon	*Lactobacillus plantarum*
	20	D2	M17	Aerobic	Amazon	*Lactococcus lactis*
	29	B2	MRS	Anaerobic	Amazon	*Weissella paramesenteroides*
	29	D1	M17	Aerobic	Amazon	*Streptococcus lutetiensis*
	48	C2	M17	Aerobic	Pacific coast	*Lactococcus garvieae*
	56	B2	M17	Aerobic	Pacific coast	*Leuconostoc mesenteroides*
	56	C2	MRS	Anaerobic	Pacific coast	*Leuconostoc mesenteroides*
Riccota cheese	3	C3	MRS	Anaerobic	Andean	*Weissella confusa*
	3	G1	M17	Aerobic	Andean	*Lactococcus lactis*
	22	B1	MRS	Aerobic	Amazon	*Leuconostoc mesenteroides*
	22	D1	M17	Aerobic	Amazon	*Enterococcus faecium*
	24	B1	MRS	Anaerobic	Pacific coast	*Lactobacillus fermentum*
	24	C1	MRS	Aerobic	Pacific coast	*Lactobacillus fermentum*
	24	E1	M17	Anaerobic	Pacific coast	*Streptococcus macedonicus*
	34	A2	MRS	Anaerobic	Andean	*Leuconostoc lactis*
	34	C1	M17	Anaerobic	Andean	*Lactococcus lactis*
	35	A1	MRS	Anaerobic	Andean	*Lactobacillus kefiri*
	36	A1	MRS	Anaerobic	Andean	*Lactobacillus plantarum*
	36	C2	M17	Anaerobic	Andean	*Lactococcus lactis*
	37	A1	MRS	Anaerobic	Andean	*Streptococcus macedonicus*
	37	D1	M17	Aerobic	Andean	*Streptococcus macedonicus*
	39	A1	MRS	Anaerobic	Andean	*Leuconostoc mesenteroides*
	39	D2	M17	Aerobic	Andean	*Lactococcus lactis*
	40	B2	MRS	Aerobic	Andean	*Leuconostoc citreum*
	40	D2	M17	Aerobic	Andean	*Lactococcus lactis*
	54	A1	MRS	Anaerobic	Pacific coast	*Lactobacillus delbrueckii*
	54	D2	M17	Aerobic	Pacific coast	*Enterococcus gallinarum*
	55	A2	MRS	Anaerobic	Pacific coast	*Lactobacillus plantarum*
	55	C2	M17	Aerobic	Pacific coast	*Streptococcus lutetiensis*
Cottage cheese	49	A2	MRS	Anaerobic	Pacific coast	*Lactobacillus rhamnosus*
	49	C1	M17	Aerobic	Pacific coast	*Lactobacillus rhamnosus*
	53	B1	MRS	Aerobic	Pacific coast	*Lactobacillus rhamnosus*
	53	C2	M17	Anaerobic	Pacific coast	*Enterococcus faecium*
Curd cheese	6	B1	MRS	Anaerobic	Andean	*Lactobacillus plantarum*
	6	E1	M17	Aerobic	Andean	*Lactococcus lactis*

	16	A1	MRS	Anaerobic	Amazon	*Lactobacillus casei*
	16	B2	M17	Anaerobic	Amazon	*Streptococcus thermophilus*
	17	C1	M17	Anaerobic	Amazon	*Enterococcus faecium*
	17	G2	MRS	Aerobic	Amazon	*Lactobacillus rhamnosus*
	28	A1	MRS	Anaerobic	Amazon	*Leuconostoc mesenteroides*
	28	D1	M17	Aerobic	Amazon	*Lactococcus lactis*
	30	B1	MRS	Anaerobic	Amazon	*Lactobacillus plantarum*
	30	C1	M17	Aerobic	Amazon	*Enterococcus faecium*
Farmer cheese	32	D2	M17	Anaerobic	Amazon	*Enterococcus faecium*
	33	B1	MRS	Aerobic	Pacific coast	*Streptococcus lutetiensis*
	33	C2	M17	Anaerobic	Pacific coast	*Streptococcus lutetiensis*
	38	C1	M17	Anaerobic	Andean	*Lactococcus lactis*
	38	D1	M17	Aerobic	Andean	*Lactococcus lactis*
	44	B2	MRS	Aerobic	Andean	*Lactobacillus paracasei*
	44	D2	M17	Aerobic	Andean	*Enterococcus italicus*
Fresh cheese	1	C2	MRS	Aerobic	Andean	*Lactobacillus plantarum*
	1	G3	M17	Aerobic	Andean	*Lactococcus lactis*
	2	A2	MRS	Anaerobic	Amazon	*Lactobacillus plantarum*
	4	A1	MRS	Aerobic	Andean	*Streptococcus macedonicus*
	4	D2	M17	Anaerobic	Andean	*Streptococcus macedonicus*
	5	C3	M17	Aerobic	Andean	*Lactococcus garvieae*
	11	C2	MRS	Aerobic	Andean	*Leuconostoc garlicum*
	11	E1	M17	Aerobic	Andean	*Lactococcus lactis*
	13	A2	MRS	Anaerobic	Amazon	*Leuconostoc pseudomesenteroides*
	13	B2	MRS	Aerobic	Amazon	*Lactococcus garvieae*
	14	E1	M17	Aerobic	Amazon	*Lactococcus garvieae*
	15	A1	MRS	Anaerobic	Pacific coast	*Leuconostoc mesenteroides*
	15	E1	M17	Aerobic	Pacific coast	*Enterococcus faecium*
	18	D1	M17	Aerobic	Amazon	*Lactococcus lactis*
	18	E1	MRS	Aerobic	Amazon	*Leuconostoc pseudomesenteroides*
	19	D2	MRS	Aerobic	Amazon	*Lactobacillus rhamnosus*
	19	E1	M17	Aerobic	Amazon	*Streptococcus lutetiensis*
	21	A2	MRS	Anaerobic	Amazon	*Lactobacillus plantarum*
	21	E1	M17	Aerobic	Amazon	*Lactococcus lactis*
	31	A1	MRS	Anaerobic	Pacific coast	*Lactobacillus plantarum*
	31	C1	M17	Anaerobic	Pacific coast	*Enterococcus faecium*
	32	B1	MRS	Anaerobic	Amazon	*Lactobacillus plantarum*
	42	A1	MRS	Anaerobic	Andean	*Pediococcus pentosaceus*
	42	D1	M17	Aerobic	Andean	*Lactococcus lactis*
	43	B2	MRS	Aerobic	Andean	*Streptococcus macedonicus*
	43	C1	M17	Aerobic	Andean	*Lactococcus garvieae*
	45	A1	MRS	Anaerobic	Andean	*Enterococcus faecium*
	45	C2	M17	Anaerobic	Andean	*Enterococcus faecium*
Goat cheese	41	B1	MRS	Aerobic	Andean	*Lactobacillus casei*

	41	C2	M17	Anaerobic	Andean	*Streptococcus thermophilus*
Mozzarella	8	D3	M17	Anaerobic	Andean	*Lactococcus lactis*
	10	G1	M17	Anaerobic	Andean	*Lactococcus lactis*
	12	D1	M17	Aerobic	Pacific coast	*Lactobacillus casei*
	12	E1	MRS	Anaerobic	Pacific coast	*Lactobacillus casei*

Table 3. Microorganisms isolated from other fermented products different of beer and cheese samples (identified by the sequencing of the 16S gene). The identities of the strains were acquired by sequencing of the 16s ribosomal gene, followed by a blast search in the NCBI GenBank Database of the isolates obtained by culture in MRS and M17 culture media of the fermented products.

Food product	Sample number	Isolate	Culture media	Culture conditions	Region	Identification
Banana vinegar	50	A2	MRS	Anaerobic	Pacific coast	*Enterococcus faecium*
	50	B2	MRS	Anaerobic	Pacific coast	*Enterococcus faecium*
	51	A1	MRS	Anaerobic	Pacific coast	*Enterococcus faecium*
	51	A2	MRS	Anaerobic	Pacific coast	*Enterococcus faecium*
Butter	57	B2	MRS	Aerobic	Pacific coast	*Lactobacillus brevis*
	57	C1	M17	Anaerobic	Pacific coast	*Lactococcus lactis*
Champús	7	K1	M17	Aerobic	Andean	*Lactococcus lactis*
	9	E1	M17	Aerobic	Andean	*Lactococcus lactis*
Fermented saussage	52	B2	MRS	Aerobic	Pacific coast	*Lactobacillus sakei*
	52.2	B2	MRS	Aerobic	Pacific coast	*Lactobacillus sakei*
	52	D1	M17	Aerobic	Pacific coast	*Weissella viridescens*
Pulcre	23	B2	MRS	Anaerobic	Amazon	*Weissella cibaria*
	23	F2	M17	Anaerobic	Amazon	*Lactococcus lactis*
Vinagre de plátano	60	B1	MRS	Anaerobic	Andean	*Enterococcus faecium*