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Exploring diversity and biotechnological potential of lactic acid bacteria from *tocosh* - traditional Peruvian fermented potatoes - by high throughput sequencing (HTS) and culturing

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Riboflavin (PubChem CID: 493570)
Folic acid (PubChem CID: 6037)
L-Tyrosine (PubChem CID: 6057)
L-Histidine (PubChem CID: 6274)
L-Lysine (PubChem CID: 5962)
L-Arginine (PubChem CID: 6322)

ABSTRACT

Lactic acid bacteria (LAB) diversity associated with *tocosh*, Peruvian traditional fermented potatoes, was for the first time investigated by culturing and high throughput sequencing (HTS) approaches. They were applied on three samples i.e. freshly harvested potatoes, one-month and eight-months production. While by culture-dependent approach a few *Lactobacillus (Lb)* species (*Lb. sakei*, *Lb. casei*, *Lb. farciminis*, *Lb. brevis*, *Lb. fermentum*) and *Leuconostoc (Ln) mesenteroides* were identified, twenty-four OTUs belonging to six LAB genera were considered in *tocosh* samples by HTS, being *Lactobacillus* dominant in all three samples. LAB predominated on fresh potatoes, while *Clostridium, Zymophilus* and *Prevotella* were the most abundant genus in 1- and 8-months *tocosh* samples. When biotechnological features were investigated, amylase and phytate-degrading abilities as well as EPS and group B vitamin (riboflavin and folate) production were exhibited by several *Lb. sakei* and *Ln. mesenteroides* strains. Safety traits of major LAB species from *tocosh* showed antibacterial activities as well as biogenic amines production capacity. The molecular inventory achieved by HTS approach provided information on LAB population composition during fermentation of this ancestral potato fermented product while culturing allowed the selection of LAB strains suitable for novel functional cultures design for the production of fermented starchy products.

1. Introduction

Household fermentation of foods has a long and very important tradition in Latin American countries (Londoño-Zapata, Durango-Zuleta, Sepúlveda-Valencia, & Moreno Herrera, 2017; Tamang, Watanabe, & Holzapfel, 2016). Andean communities have managed to preserve native crops as well as their production, harvesting and storage during centuries. Among them, potatoes, originated approximately 8000 years ago in the South American Andes, Perú lodging one of the most important reservoirs of varieties and wild relatives (Goldner, Pérez, Pilosof, & Armada, 2012; Mosso, Lobo, & Sammán, 2016; Velásquez-Milla, Casas, Torres-Guevara, & Cruz-Soriano, 2011). Numerous ingenious ways of preserving potatoes in order to maintain adequate stocks for survival have been developed, such as sun-drying or natural freeze-drying to obtain white or black *chuño* and fermentation to obtain *tocosh*.

Potatoes *tocosh* is an ancestral fermented food product that is

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still prepared in small communities from the highlands of Central Peruvian Andes by local peasants (Horkheimer, 1973). The traditional preparation method consists of digging a well (0.70 × 1.50 m deep) in the ground near a water spring in which large amounts of potatoes (normally discarded potatoes) are placed between straw layers. Rocks are used to cover the pile in order to prevent the tubers to be washed away by the slight water current that passed through the ditch; the potatoes are then left to ferment in this running water up to 12 months. After this time, potatoes suffer an enzymatic browning (Zvitov-Ya'ari & Nussinovitch, 2014) and are laid in a dry shaded area to allow the water to drain. The obtained product is kept for consumption, sale or most commonly, as a sun-dried and ground fine flour-type product that is used to prepare different broths, stews and "mazamorra" which is a semi-liquid food with thick consistency (De Moreno de LeBlanc, Todorov, Vignolo, Savoy de Giori, & LeBlanc, 2014). From a microbiological point of view, only preliminary studies have been performed demonstrating that tocosh results from microbial fermentation, mainly by lactobacilli. Besides being an important staple food for local population, the compounds generated by these beneficial microorganisms are thought to be responsible for the large diversity of medicinal properties attributed to this product as such being known as the "natural antibiotic of the Incas". Although no scientific articles supporting these claims were found, its probiotic potential was demonstrated using an experimental animal model and compared with a recognized probiotic *Lactobacillus acidophilus* LA-5® strain (Prentice & Milka, 2005). Lactic acid bacteria (LAB) are usually involved in traditionally fermented food of vegetal origin and many LAB species have been described as vitamin producers or phytate degraders (Anastasio et al., 2010; Juarez del Valle, Laiño, Savoy, de Giori, & LeBlanc, 2014; Ruiz-Rodríguez et al., 2016).

Recently environmental and food microbiology have benefited from the advances in molecular biology and adopted novel strategies to detect, identify, and monitor microbes. An in-depth study of the microbial diversity in food can now be achieved by using high-throughput sequencing (HTS) approaches after direct nucleic acid extraction from the sample to be studied; the current scenario of this metagenomic approach to study food microbiota was described by Ercolini (2013). Therefore, the aim of this study was to evaluate LAB populations present in *tocosh* and to disclose their biotechnological potential for future applications. Fresh potatoes and fermented samples from two wells corresponding to two storage times were analysed by combining both, culture-dependent and HTS approaches.

2. Material and methods

2.1. Bacterial strains and growth conditions

LAB reference cultures used in this work were supplied by the Spanish Type Culture Collection (CECT) as follows: *Lactobacillus sakei* subsp. *sakei* CECT 906^T, *Leuconostoc mesenteroides* subsp. *cremoris* CECT 872^T, *Leuconostoc mesenteroides* subsp. *dextranicum* CECT 912^T, *Leuconostoc mesenteroides* subsp. *mesenteroides* CECT 219^T, *Lactobacillus amylophilus* CECT 4133^T. LAB strains were routinely grown on MRS (De Man, Rogosa and Sharpe) medium (Oxoid) at 28 °C and stored in growth liquid medium containing 20% (v/v) glycerol at −80 °C. *Listeria monocytogenes* FBUNT (Facultad de Bioquímica, Quíimica y Farmacia, UNT, Argentina) and *Bacillus subtilis* 168 (PROIMI-CONICET) were grown overnight on Trypticase Soy broth (TSB, Britania, Argentina) at 30 °C.

2.2. Sample processing, microbiological analysis and LAB isolation

Potato *tocosh* samples were obtained from ground wells traditionally prepared by local producers from the community of Tambogán, (Huánuco, Perú) located at 2500 m above sea level (Fig. 1). The region presents average annual temperatures of 12 °C, rainy summers and winters with strong frost, temperature during the day being largely variable (22 to −3 °C) but keep constant along the year. Due to difficult access to this place, all samples were collected at once in Summer 2012, corresponding to freshly harvested potatoes (before placing in the ground well), 1-month and 8-months *tocosh* storage wells. Samples (5 g) were analysed as previously described for microbial counts (Elizaquível et al., 2015): total mesophilic counts on Plate Count Agar, incubated aerobically at 30 °C for 72 h; LAB on MRS containing glucose (MRS), maltose (MRS-M) or starch (MRS-S) at 0.5% (w/v) and Yeast Glucose Lactose Peptone (YGLP), incubated anaerobically at 30 °C for up to 7 days; total yeasts and molds on Yeast and Molds agar, incubated aerobically at 30 °C for 72 h. Counts were performed in triplicate. For each sample, up to six colonies per plate and LAB medium, representing different morphologies, were randomly picked from plates with 30–300 colonies and sub-cultured on the corresponding medium. Isolates that were Gram positive and catalase negative were considered as presumptive LAB and were stored at −20 °C in the same liquid media containing 20% (v/v) glycerol for further analysis. Samples pH was determined from homogenized samples using a digital pH meter (PT-10 Sartorius).

2.3. Culture-dependent analysis of LAB populations

2.3.1. PCR-based LAB identification

DNA extraction and identification of pure isolates into species was approached following a three steps schedule as previously described (Elizaquível et al., 2015), with some modifications: isolates from each ISR group, were subjected to RAPD-PCR analysis using universal primers P2 (5′-GATCGGACGG-3′) and P16 (5′-TCGCCAGCCA -3′) as described by Samaržija, Sikora, Redzepović, Antunac, & Havranek, 2002, In addition, the 16S rRNA sequences of selected isolates representing the different clusters were compared with the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/) for species identification.

2.3.2. Cluster analysis of ISR-PCR and RAPD-PCR electrophoretic profiles

Digitized images were converted, normalized, analysed and combined using the Software package BioNumerics 4.61 (Applied Maths, Kortrijk, Belgium). Identification of profiles was carried out by comparison with a database previously generated with the aid of the BioNumerics software, containing ISR and RAPD profiles corresponding to 132 reference strains (Chenoll, Macián, Elizaquível, & Aznar, 2007).

2.4. Culture-independent analysis of bacterial populations

2.4.1. Tocosh DNA isolation, 16S rRNA gene amplification and pyrosequencing

For DNA isolation, 10 ml of each homogenized sample was taken from the upper liquid phase and centrifuged (5000 × *g*, 10 min). Total DNA was extracted using the Bacterial DNA preparation kit (Jena Biosciences, Germany) according to the manufacturer's instructions.

Amplicon library preparation and pyrosequencing were carried out by LifeSequencing Inc. (Valencia, Spain). The DNA isolated from *tocosh* samples was used as template for the amplification of the V3–V5 hypervariable region of the bacterial 16S rRNA genes with primer set 357F/926Rb (Sim et al., 2012). Amplicon library preparation and pyrosequencing was carried out by LifeSequencing Inc. (Valencia, Spain) as previously described in Elizaquível et al. (2015).