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Characterization of lactic acid bacteria isolated from traditional Ecuadorian fermented foods

Caracterización de las bacterias ácido lácticas aisladas de alimentos fermentados tradicionales del Ecuador

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Abstract

Ninety one samples of traditional fermented foods were collected from 20 Ecuadorian provinces. In order to identify strains with potential use as starter cultures in fermented foods, 119 lactic acid bacteria (LAB) strains were isolated and characterized by phenotypic tests and 16S ribosomal DNA (rDNA) sequencing. Forty seven percent of isolates were identified as facultative heterofermentative *Lactobacillus sp*. the next predominant groups (31%) were homofermentative cocci belonging to the genera *Pediococcus, Lactococcus* and *Enterococcus*; Finally, 11% were obligate heterofermentative *Lactobacillus sp.* and 11% heterofermentative cocci (*Weissella* and *Leuconostoc*). Several isolates showed Exopolysaccharide and bacteriocin production as well as caseinolytic activity. These properties could have interesting applications in food industry.

Keywords. Lactic Acid Bacteria, 16S ribosomal DNA, Artisanal Fermented foods.

Resumen

En 20 provincias ecuatorianas, se colectaron 91 muestras de alimentos fermentados preparados de forma artesanal con el objetivo de identificar cepas con uso potencial en la producción de alimentos fermentados. Se aislaron 119 cepas que fueron caracterizadas a través de pruebas fenotípicas y secuenciamiento del ADN ribosomal 16S. Del total de cepas aisladas, 47 % fueron *Lactobacillus sp*. heterofermentadores facultativos, 31% fueron cocos homofermentadores pertenecientes a los géneros *Pediococcus, Lactococcus* y *Enterococcus*, 11% fueron *Lactobacillus sp.* heterofermentadores obligados y el 11% restante cocos heterofermentadores de los géneros *Weissella* y *Leuconostoc*. Algunas cepas presentaron ciertas propiedades con posible aplicación en la industria alimentaria como actividad caseinolítica y la producción exopolisacárido y bacteriocinas.

Palabras Clave. Bacterias Acido Lácticas, ADNr 16S, Alimentos fermentados artesanales

Introduction

Lactic acid bacteria (LAB) are fermenting gram-positive, non-sporulating, cocci or rods, which produce lactic acid in their fermentation [1]. Most common LAB species used in food fermentation belong to the genera *Lactobacillus, Leuconostoc, Pediococcus* or *Streptococcus* [2].

The consumption of LAB's in fermented foods is ubiquitous and ancient part of the human culture because it helps to preserve food and provides variety of sensorial and rheological characteristics that make them unique [3]. Food fermentation is also known to enhance digestibility of some substances such as lactose, cyanogenic glycosides such as linamarin [4] and may reduce undesirable substances present in raw foods such as phytates, tannins, and polyphenols [5]. Some reports claim that fermented foods may increase mineral bioavailability, production of antioxidants and omega-3 polyunsaturated fatty acids, and even improvements in immune functions [6, 7] Majority of fermented foods consumed are still produced traditionally in Ecuador and at small-scale where the fermentation occurs without the addition of starter cultures [8] and with no control over patho-

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genic microorganisms [9].

Ecuador has more than 15 culturally different ethnic groups resulting in a diverse range of culinary traditions, including fermented products. This report characterizes LAB species from some of the most popular traditional fermented foods in Ecuador such as, cheeses, spicy sauces, sausages, vinegar and alcoholic beverages (Chichas).

Materials and Methods

Sample collection

A total of 91 samples from fermented foods were collected in three geographical regions distributed in 20 provinces of Ecuador, Coast region (n=31), Andean region (n=32), and Amazon region (n=35). Samples of traditional beers known as chicha prepared with corn (n=3), a fruit of the palm *Bactris gasipaes* locally known as chonta (n=4) orcassava (n=4) were collected. Additionally, we collected samples from other traditional products such as vinegar (n=3), eggnog (n=1) cheeses (n=69), and sausages (n=7). All the samples were transported and preserved to 4°C.

Cultivation and isolation of LAB species

Twenty grams of each sample were homogenized with 180 ml sterile 2% trisodium citrate solution ($Na_3C_6H_5O_7$) and each homogenate was diluted (10^{-2} to 10^{-7}) using sterile 0.85% NaCl and 1ml of each dilution was plated on 1 MRS agar plates pH 5.7 (Merck, Germany) and M17 agar supplemented with 0.5%, glucose pH 7.2 (Merck, Germany), cultures were carried out in duplicates. Dilutions 10^{-4}, 10^{-5}, 10^{-6}, and10^{-7} were incubated at 37°C, and dilutions 10^{-2}, 10^{-3}, 10^{-4}, and10^{-5} were incubated at 45°C for 3-5 days under aerobic and anaerobic conditions. One millilitre of 10^{-1} dilution was inoculated in 9 ml of three different culture media: MRS, M17 supplemented with 0.5% glucose (GM17) and sterile skimmed milk. All tubes were incubated anaerobically at 30°C and 45°C for 24 h, plated on MRS and GM17 agar and incubated anaerobically at 30°C and 45°C for 3 to 5 days. Twenty to 100 colonies were randomly selected from each plate and further characterized. A total of 119 gram positive and catalase negative isolates were transferred to MRS or GM17 broth containing 15% of sterile glycerol and stored at –80°C.

Biochemical activities

To test for acidification activity, one millilitre of overnight culture was transferred to 9 ml of sterile milk and incubated at 37°C and 45°C for 18hours and pH of sterile milk was assessed using pH strips (Macherey-Nagel-Düren) before and after incubation.

To characterize bacterial growth curves at 25°C, 37°C and 45°C, 3 tubes containing 5ml of either MRS or GM17 were inoculated with 1ml of overnight culture and optical densities were monitored every hour for 10 hours using an spectrophotometer (λ=600 nm).

Caseinolytic activity was performed using a modified version of the procedure describe by [10]. Bacteria were collected from the Milk Citrate Agar (MCA) plates and resuspended in ammonium acetate buffer (100 mM, pH 6.8) to obtain a density of 3 x 10^8 cells per ml. Cell suspensions were mixed with the same volume of β-casein solution (5 mg/ml) in ammonium acetate buffer (100 mM, pH 6.8) (Sigma Chemie GmbH, Deisenhofen, Germany). The suspensions of cells and β-casein were incubated for 3 hrs.at 37°C or 45°C and then centrifuged to remove the cells.

Degradation of β-casein was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [11]. The supernatants were taken and mixed with the sample loading buffer (125 mM TrisHCl, pH 6.8, 10 mM EDTA, 4% SDS, 25% glycerol, 5% 2-mercaptoethanol and 0.07% bromophenol blue) in a 1:1 volume ratio. Prior to loading, samples were heated at 100°C for 2 min and analyzed on 15% acrylamide gels. Gels were stained with Coomassie brilliant blue R250 (Serva, Heidelberg,Germany) and distained in a mix of methanol (20%) and acetic acid (7%).

Exopolysaccharide production (EPS) was performed as [12], bacterial strains were cultivated in MSE (Mayeux Sandino Ellik) agar with 2% w/v glucose at 37°C for 18h and EPS was detected as an elastic and slim y texture of the colonies.

Detection of bacteriocin activity was carried out using agar-well diffusion assay [13]. Semisolid GM17 or MRS (0.7% agar w/v), containing lactococci or lactobacilli indicator strains (*Lactococcus lactis* subsp. *lactis* BGMN1-596 and *Lactobacillus paracasei* subsp. *paracasei* BGBUK2-16/K4), was overlaid on solid GM17 and MRS (1.5% agar w/v) plates, respectively. Wells were made in the lawn of hardened semisolid agar. Aliquots (50 μl) of supernatant from overnight cultures (16 h) were poured in the wells and plates were incubated overnight at 30°C. A clear zone of inhibition around the well was recorded as positive signal for antimicrobial activity.

Genotypic characterization

Genomic DNA was released from cells using the heat shock/boiled-cell method [14], 5 isolated colonies from an axenic culture were resuspended in 300μl of sterile water and heated at 100°C for 10 min. Suspension was centrifuged at 13,000 rpm for 3 min and stored immediately at - 20°C overnight. Amplification of 16S rRNA gene was performed using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'- G GTTACCTTGTTACGACTT-3') [15]. PCR mixture consisted of 1X PCR Buffer (Promega), 0.25 mM of each dNTP, 0.2 uM of each primer, 0.6 U GoTaq DNA polymerase (Promega, Madison USA), 5 ul of DNA extracts, and Milli-Q water to a total volume of 25 μl. PCR conditions were as follows: denaturation step at 94°C for

1 min followed by 30 amplification cycles (94°C for 30 s, 50°C for 30 s, 72°C for 30 s) and a final extension at 72°C for 10 min.

Amplicons were analyzed by electrophoresis in 1% agarose gel. PCR products were directly sequenced in both directions by Functional Biosciences USA. The sequences were treated using Pregap and Gap softwares included in the Staden Package [16] and were compared to entries of homologous sequences in the GenBank and Ribosomal Database Project.Sequence alignments were done utilizing ClustalW software [17]. Neighbour-Joining analysis was performed using MEGA version 4.0 software [18] with the Kimura-2 parameter model and using uniform rates among sites.

Results and Discussion

A total of 114 isolates were obtained from Ecuadorian traditional fermented products (Table 1). Majority of the isolates belonged to genus *Lactobacillus* (n=68) followed by *Enterococcus* (n=15), *Pediococcus* (n=13), *Lactococcus* (n= 10), *Leuconostoc* (n=7) and *Weisella* (n=6) accession numbers: JQ446453-JQ446571 (Figure 1-6). These results are in agreement with previous reports indicating that *L. plantarum* is the dominant species in vegetal and milk fermented foods [19–22]. *L. plantarum* strains occur in late phases of fermentation [20] these bacteria are found at the end of many spontaneous lactic fermentations such as silage and vegetable fermentations [23]. *L. plantarum* has been associated to desirable biochemical properties [24] and it is the most frequent species found in different foods and silages [25–27]. In our study *Pediococcus* was mainly isolated from sausages; this bacterium has been used starter culture for the production of sausages thanks to its ability to grow under reduced water activity [28, 29]. *Pediococcus* was also isolated from cheese (Table 1) however this bacterium is not common in dairy products [30].

On the other hand, *Leuconostoc* was isolated mainly from traditional beers prepared with corn, cassava, and chonta. This bacterium has been found in kimchi and sauerkraut [30]. Members of the genus *Weissella* were isolated from cheese (Table 1), however these bacteria have been reported in fresh vegetables, sugar cane, meat samples, and also from clinical samples from animals and humans [31]. *Lactococcus* was isolated from cheese and sausage and it has been reported in plants, vegetables, and cereals products [32, 33]. *Enterococcus* species were isolated from six products suggesting fecal contamination. However, enterococci largely occur in many cheeses, and are considered to be part of their typical microbiome [34].

Three *Lactobacillus* strains *L. curvatus* (n=1), *L. rhamnosus* (n=2), and two *Lc. lactis* strains were identified as high acid producers (Δ pH>2), a *Lc. lactis* strain isolated from cheese had the highest acidifying activity (Δ pH>2.5 in 18 hrs of incubation). Acidification to pH levels lower than 4.2 constitutes a major food safety factor [35].

All *Pediococcus* strains, 92% of *Lactobacillus* spp. strains, 50% of *Weisella* strains, and 80% of *Lactococcus* and *Enterococcus* sp. were found to produce proteinases. Proteolytic activity of dairy lactic acid bacteria is essential for the bacterial growth in milk and it is also responsible for the development of organoleptic properties of different fermented milk products [36, 37]. In this study, 83% of strains were found to produce proteinases indicating that most of these isolates could be used in fermented dairy foods. Proteinase activity is a characteristic widely found between lactic acid bacteria, particular lythose isolated from milk products [38]. In contrast, none *Leuconostoc* isolates was proteinase positive.

Eight percent of isolates produced EPS in MSE agar: *Ln.mesenteroides* (4), *L. fermentum* (2), *L. plantarum* (2), and *Enterococcus lactis*. (1)*Leuconostoc mesenteroides* has been previously reported to increase viscosity in Mexican alcoholic beverage called pulque [39, 40] another fermented products [41].

Two bacterial species isolated from cheese *Lactobacillus* (QX2) and *Leuconostoc* (QX5an) isolate produced bacteriocins. Bacterial growth inhibition due to low pH was rule out by neutralizing the media to pH 6.5 before testing against the organisms. Various bacteriocins produced by *L. plantarum* have been described, i.e. plantaricin A [42] and plantaricin B [43]. Several plantaricins have been reported to inhibit *Listeria* spp. and other gram positive bacteria [44, 45].

On average, isolates incubated at 37°C and 45°C reached the stationary phase within 10 and 8 h respectively ($\approx$ 0.7 OD at 600 nm). While isolates incubated at room temperature were still in exponential after 10 h incubation.

Conclusions

This study showed that culturable microbiota associated to some Ecuadorian traditional acidic fermented foods encompassed *Lactobacillus* 57%, *Enterococcus* 13%, *Pediococcus* 11%, *Lactococcus* 8%, *Leuconostoc* 6%, and *Weissella* 5%. Most LAB strains were facultative heterofermentative *Lactobacillus* showing 99% nucleotide identity to either *L. plantarum* or *L. fermentum*. Other dominant bacteria were *Pediococcus*, *Lactococcus* and *Weissella* which belonged to homofermentative cocci group.

Nucleotide sequences from Ecuadorian isolates did not show any tendency to group with Andean or Latin - American isolates. This suggests that the origin of these bacteria may be broad, however this may not rule out local metabolic diversity due to acquisition of laterally transferred genes.

There is limited information about Ecuadorian traditional fermented foods except for two studies carried out in

Product	Identified Species	Number of Isolates
Vinegar	*Lb. fermentum*	1
	Lb. plantarum	2
Traditional beer (Chicha)	*Lb. plantarum*	10
	Lb. fermentum	5
	Lb. paracasei	1
	E. lactis	1
Sausage	*Lb. sakei*	1
	Lc. garviae	2
	P. pentosaceus	4
	Lc. lactis	2
Traditional Cheeses	*Lb. plantarum*	21
	Lb. rhamnosus	7
	Lb. fermentum	7
	Ln. mesenteroides	6
	Lc. lactis	6
	W. paramesenteroides	6
	E. faecalis	5
	P. pentosaceus	5
	Lb. casei	4
	E. italicus	3
	P. acidilactici	3
	E. durans	3
	E. faecium	2
	Lb. Paraplantarum	2
	P. stilesii	1
	Lb. zeae	1
	E. casseliflavus	1
	Lb. pentosaceus	1
	Lb. curvatus	1

Table 1: Ecuadorian fermented products and bacterial species cultured from them.

yellow rice [46, 47] and a recent one on cocoa beans [48]. This report provides important information about fermenting microorganisms in some of the most popular fermented products elaborated according to Ecuadorian folk traditions.

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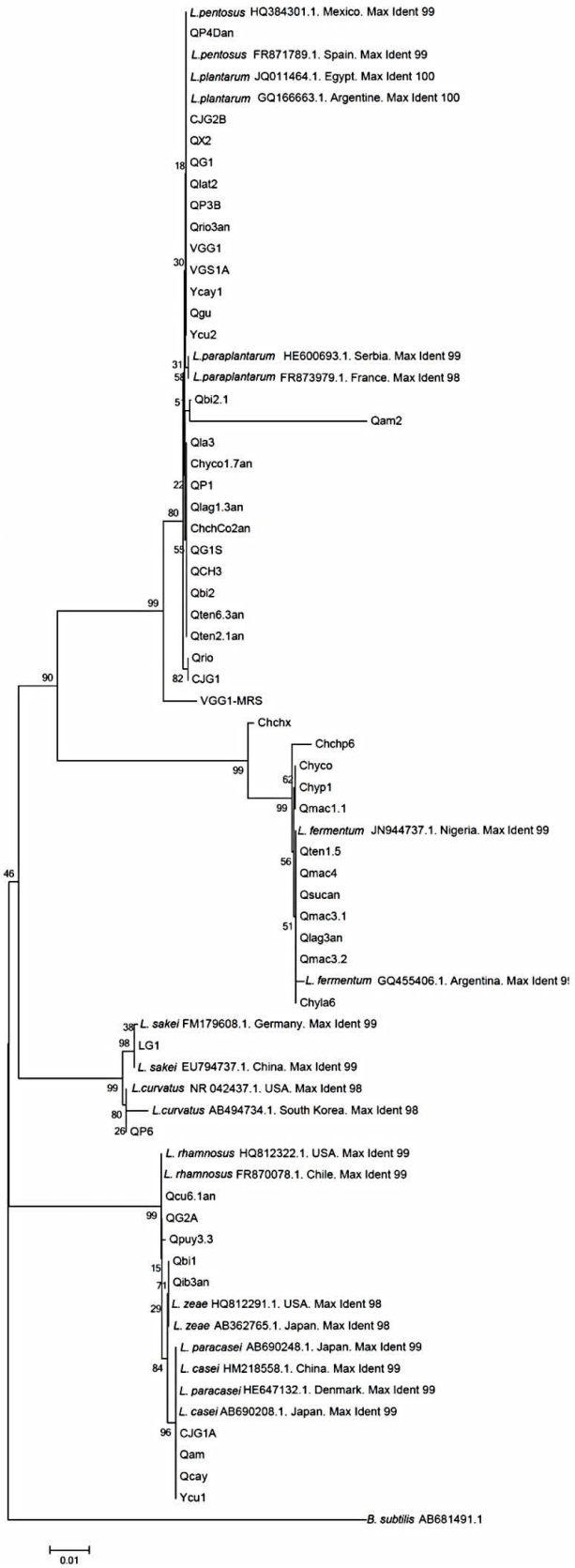

Figure 1: Phylogenetic tree of 16S rDNA sequences *Lactobacillus* strains using Neighbor-joining method. QP4Dan, CJG2B, QG1, QX2, Qlat2, QP3B, VGG1, QRio3an, VGS1A, Ycay1, QGu, Ycu2, Qbi2.1, Qla3, Chyco1.7an, QP1, Qlag1.3an, ChchCo2an, QG1S, QCh3, QBi2, Qten6.3an, QTen2.1an, QRio, CJG1, VGG1, Chchx, Chchp6, Chyco, Chyp1, Qmac1.1, Qten1.5, Qmac4, QSuc an, Qmac3.1, Qlag3an, Qmac3.2, Chyla6, LG1, QP6, Qcu6.1an, QG2A, Qpuy3.3, Qbi1, Qib3an, CJG1A, QAm, Qcay, YCu1, are isolates described in this paper, other sequences were obtained from GenBank. The corresponding bootstrap values (100 replications) are shown on each branch.

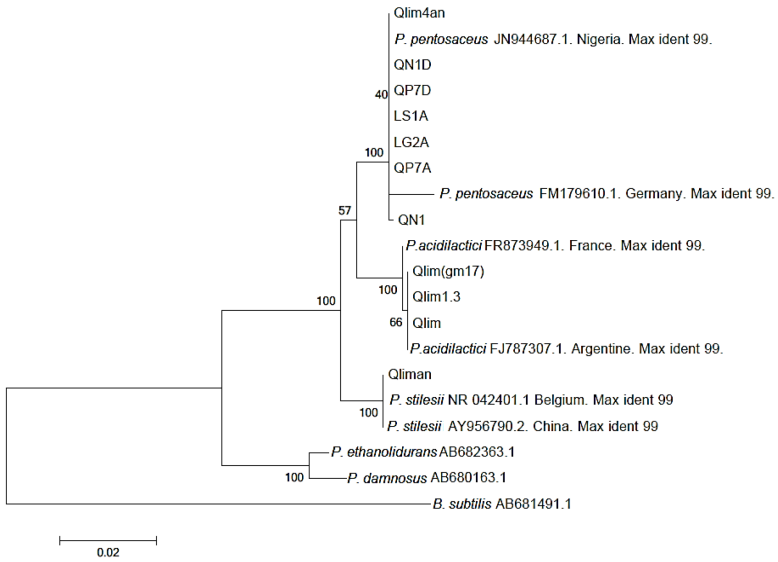

Figure 2: Phylogenetic tree of 16S rDNA sequences *Pediococcus* strains using Neighbor-joining method. QN1D, Qlim4an, QP7D, LS1A, LG2A, QP7A, Qlim (gm17), Qlim 1.3, Qlim, Qliman are isolates described in this paper, other sequences were obtained from GenBank. The corresponding bootstrap values (100 replications) are shown on each branch.

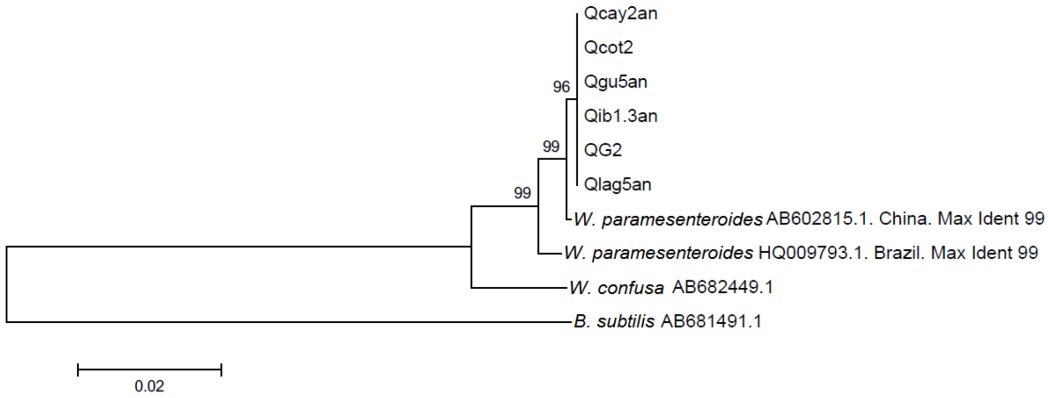

Figure 3: Phylogenetic tree of 16S rDNA sequences *Weissella* strains using Neighbor-joining method. Qcay2an, Qcot2, Qgu5an, Qin1.3an, QG2, Qlag5an are isolates described in this paper, other sequences were obtained from GenBank. The corresponding bootstrap values (100 replications) are shown on each branch.

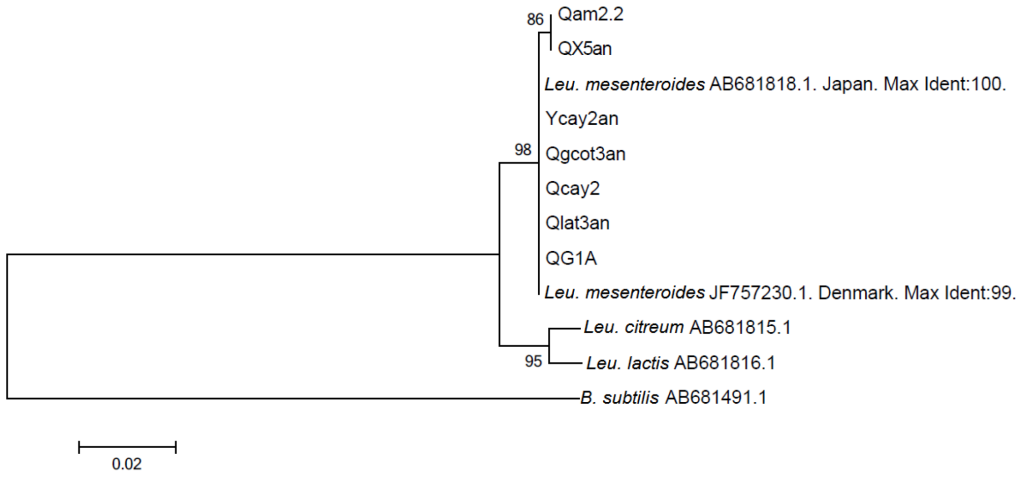

Figure 4: Phylogenetic tree of 16S rDNA sequences *Leuconostoc* strains using Neighbor-joining method. Qam2,2, QX5an, Ycay2an, Qgcot3an, Qcay2, Qlat3an, QG1A, are isolates described in this paper, other sequences were obtained from GenBank. The corresponding bootstrap values (100 replications) are shown on each branch.

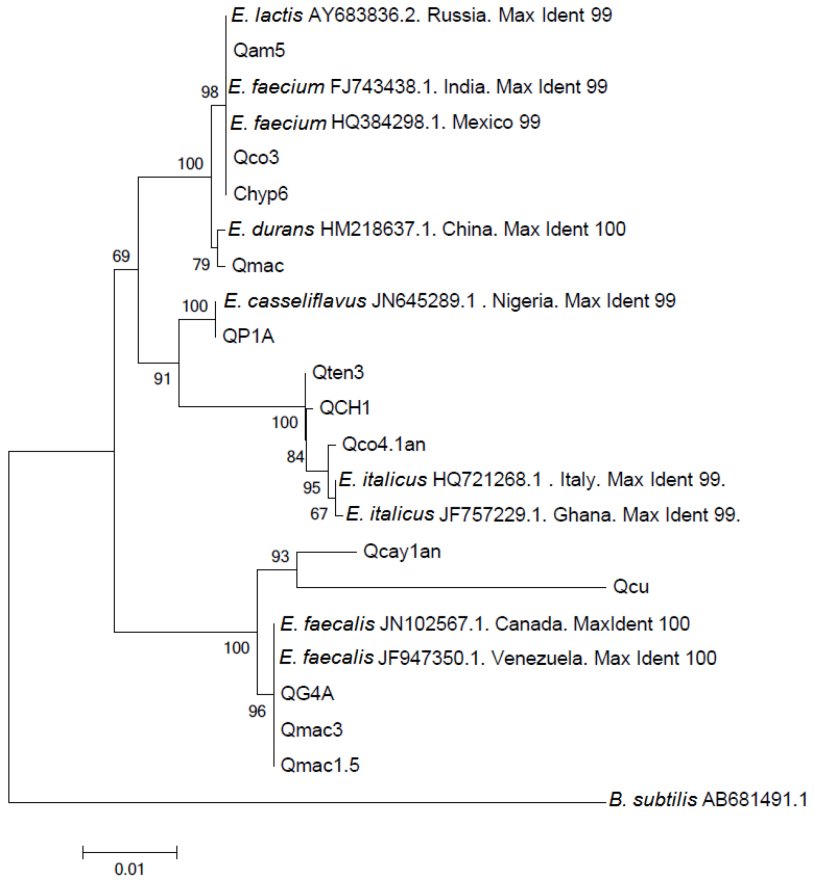

Figure 5: Phylogenetic tree of 16S rDNA sequences *Enterococcus* strains using Neighbor-joining method. Qam5, Qco3, Chyp6, Qmac, QP1A, Qten3, QCh1, QCo4.1an, QG4A, Qmac3, Qmac1.5 are isolates described in this paper, other sequences were obtained from GenBank. The corresponding bootstrap values (100 replications) are shown on each branch.

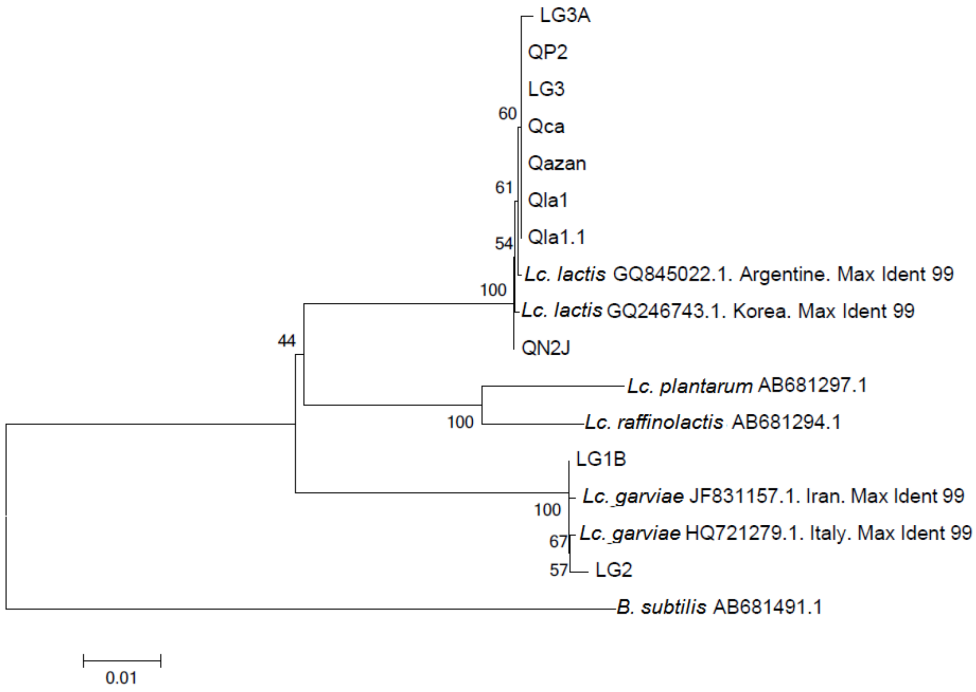

Figure 6: Phylogenetic tree of 16S rDNA sequences *Lactococcus*, strains using Neighbor-joining method. LG3A, QP2, LG3, QCa, Qazan, Qla1, Qla1.1, QN2J, LG1B, LG1, are isolates described in this paper, other sequences were obtained from GenBank. The corresponding bootstrap values (100 replications) are shown on each branch.

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