

Microflora of Naturally Fermented Table Olives and Characterization of Their Lactic Acid Bacteria

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Abstract: Table olive fermentation is one of the oldest applications in the Anatolia and table olive consumption plays important role in human nutrition. In this study, 8 traditionally produced olive samples and one industrially produced table olive sample were obtained from Aydın province. Microbial populations were determined by standard plate counts for mesophilic bacteria, lactic acid bacteria (LAB), Enterobacteriaceae, Staphylococci, mold and yeasts. Forty one representative lactobacilli were characterized by phenotypic techniques. Among them 34 of isolated strains have good growth abilities both at 15°C and 6.5% salt concentration. Additionally, those lactobacilli strains have homofermentative character and these are desired properties for table olive starters. For species level identification, four representative strains selected from sugar fermentation profiles were characterized by 16S rDNA sequencing. Three of them matched with *Lb. plantarum* and one of them matched with *Lb. pentosus*. These strains produced conjugated linoleic acid between 12-20 µg/ml with presence of linoleic acid. Finally, those LAB strains with desired technological properties can be used in controlled industrial table olive productions in the future.

Anahtar Kelimeler: identification, CLA, *Lactobacillus*, starter cultures, fermentation

Doğal Fermente Sofralık Zeytinlerin Mikroflorası ve Laktik Asit Bakterilerinin Karakterizasyonu

Öz: Sofralık zeytin fermantasyonu Anadolu'nun en eski uygulamalarından biridir ve sofralık zeytin tüketimi insan beslenmesinde önemli rol oynamaktadır. Bu çalışmada, geleneksel olarak üretilen 8 zeytin örneği ve bir adet endüstriyel olarak üretilen sofralık zeytin örneği Aydın ilinden temin edilmiştir. Mikrobiyal popülasyonlar, mezofilik bakteriler, laktik asit bakterileri (LAB), Enterobakteri, Stafilokok, küf ve mayalar standart plaka sayım yöntemleri ile belirlenmiştir. Kırk bir temsili laktobasil, fenotipik tekniklerle karakterize edilmiştir. İzole edilmiş laktobasil türlerinin 34'ü, hem 15°C'de hem de % 6,5 tuz konsantrasyonunda iyi büyüme yeteneklerine sahiptir. Ayrıca, bu laktobasil suşları homofermentatif karaktere sahiptir ve bunlar sofralık zeytin starterleri için istenen özelliklerdir. Tür düzeyinde tanımlama için, şeker fermantasyon profillerinden seçilen dört temsili suş, 16S rDNA sekanslaması ile karakterize edilmiştir. Bunlardan 3 tanesi *Lb. plantarum* ile, biri *Lb. pentosus* eşleşmiştir. Bu suşlar, linoleik asit varlığında 12-20 µg/ml arasında konjuge linoleik asit üretmişlerdir. Sonuç olarak, istenilen teknolojik özelliklere sahip bu LAB suşları, ileride kontrollü endüstriyel sofralık zeytin üretiminde kullanılabilirler.

Keywords: tanımlama, CLA, *Lactobacillus*, starter kültürler, fermantasyon

INTRODUCTION

In general, table olive production is based on fermentation and these food products have an important economic value especially in our country and Mediterranean countries for centuries. In recent years, increased consumer attention has existed for table olives due to the demand for healthy foods around the world. Beside main nutritional components, they have specific health promoting components such as essential amino acids, unsaturated fatty acids, phenolics, tocopherols, triterpenes and other antioxidant substances (Kailis and Kiritsakis, 2017). In addition those properties, their native lactic acid bacteria can contribute probiotic properties (Bautista-Gallego et al., 2013). Many scientists describe table olives as the “food of the future” (Bonatsou et al., 2017).

Olive fruit is a drupe with high oil (12–30%) and low sugar content (2.6–6.0%) and contains oleuropein (Erten et al., 2016). Oleuropein is responsible for characteristic bitterness and it is hydrolyzed by alkali treatment and reduced by fermentation (Heperkan, 2013).

Common table olive classification is based on colour (green olive, black olive, turning color) and the processing technique (natural olives, treated olives, darkened olives by oxidation, dehydrated and/or shrivelled olives) (IOOC, 2004). Common table olive trade processes are brining of olives without lye treatment, dry salt application, which is also known as sele, and removal of water with suitable drying methods (IOOC, 2004).

Microbial groups consisted of mainly lactic acid bacteria (LAB) and yeasts in the table olive fermentation. *Lactobacillus*, *Enterococcus*, *Pediococcus*, *Leuconostoc* and *Lactococcus* are the main LAB genera of the table olives (Hurtado et al., 2008; Hurtado et al., 2011; Hurtado et al., 2012; Heperkan, 2013). Sequential microbial growth is

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considered during fermentation. Initially, main microorganisms are yeasts, molds, and aerobic Gram-negative bacteria (Hurtado et al., 2012; Erten et al., 2016). It should be short as possible to inhibit spoilage. Then, acidity is improved due to the spontaneous fermentation of autochthonous lactic acid bacteria. When the total acidity approaches 0.15%–0.30% (w/v) in lactic acid, second phase is initiated. In this phase, Gram negative organism population decrease and LAB population such as *Leuconostoc* spp., *Pediococcus* spp., and *Lactococcus* spp. increase and they contribute to acidity development. In the following phase, various *Lactobacillus* spp. become dominant because they can show higher resistance to acid environment. Fermentation continues until the carbohydrate sources are exhausted. (Botta and Cocolin, 2012). Time period from the end of fermentation until packaging time can be considered as last phase. In this period, *Propionibacterium* species can be observed also. During fermentation, yeasts may play role in improvement of organoleptic properties and can be desirable. However, they can also cause some spoilage cases (Arroyo-Lopez et al., 2012).

In the past, the main role of fermentation was preservation. Nowadays, there has been increase in consumer demand for fermented foods and it is especially associated with their health benefits and functional food (Bautista-Gallego et al., 2013). Conjugated linoleic acid (CLA) production can be one of the desirable properties for fermenting bacteria. CLA can be described as a mixture of positional and geometrical isomers of linoleic acid with conjugated bonds and it has various health promoting activities such as anti-carcinogenic, anti-obesity, anti-cardiovascular and anti-diabetic, enhancing immune function, reducing body fat (Gorissen et al., 2015). It can be biotransformed by various LAB species (Terán et al., 2015; Torlak et al., 2016). One of the main microbial group in table olive fermentation is LAB and they can contribute CLA production during fermentation. Microbial flora of various types of table olives have been studied for years (Heperkan, 2013; Bautista-Gallego et al., 2013; Doulgeraki et al., 2013; Bleve et al., 2014). Although there exist significant table olive productions and consumptions in Turkey, there are limited studies on their LAB flora (Sarıkaya et al., 2008; Sozbilen and Baysal, 2016). The aim of this work is to investigate microbial properties of traditionally fermented table olives and to characterize their LAB flora by considering phenotypic, genotypic, biochemical and technological properties for development of defined cultures for table olive productions.

MATERIAL AND METHODS

Olive samples

In total, 15 samples were analyzed in the experiments. Eight traditionally produced olive samples and one industrially produced table olive sample were obtained by local market in Aydın province (Table 1) and 6 of them have also their brine solutions. Five of them were black olive samples and 4 of them were green olive samples. Two of black olive samples were called as “Sele” type table olive variety and produced by using dry salt. Samples were taken into sterile glass jars. They were stored at 4°C and analyzed in 24 h. Only one sample was industrially produced (O9) and analyzed for comparison purposes.

Table 1. Table olive samples

Sample	Sample Information
O1	Green olive
O1 brine	
O2	Black olive
O2 brine	
O3	Black olive (with oil)
O4	Black olive (Sele type)
O5	Black olive (Sele type)
O6	Green olive
O6 brine	
O7	Black olive
O7 brine	
O8	Dark green olive
O8 brine	
O9	Green olive
O9 brine	

Chemical Analysis

The pH for both in the pulp and packing brine of table olives was measured directly with an Inolab pH 7110. The pH of table olive samples was measured in the first decimal dilution of the sample homogenate prepared for microbiological analysis. Titration acidity was analyzed by using 0.1 N standardized solution of NaOH with phenolphthalein indicator and it is expressed by means of percentage (w/v) of lactic acid.

Microbial Analysis

Ten g of each table olive samples and 10 mL of their brines were analyzed by serial dilution plating methods. The media and conditions are as follows: Plate Count Agar (PCA; Merck) for total aerobic mesophilic counts incubated at 30°C for 48 h; de Man Rogosa Sharp agar (MRS; Merck) for LAB incubated at 37°C for 4 days in anaerobiosis (Qxoid anero jar and kits); Violet Red Bile Agar (VRBA; Merck) for

Enterobacteriaceae incubated at 37°C for 18-24 h; Baird-Parker agar base (BPA; Merck) with Egg Yolk Tellurite emulsion (Sigma) for Staphylococci incubated at 37°C for 48 h; Potato Dextrose Agar (PDA; Merck) for molds and yeasts incubated at 30°C for 72 h.

Isolation and Characterization of Lactobacilli

Totally, 200-300 colonies were obtained from MRS agar plates and transferred into MRS broths. Purification was done by streaking and sub-culturing at 37°C repeatedly. Isolated strains pre-identified by catalase test, Gram staining and cell morphology. Physicochemical properties such as homofermentative behavior, arginine hydrolysis, growth ability for different NaCl concentrations (4%, 6.5% and 10% NaCl) and temperatures (15°C and 45°C) were analyzed as described by Bulut et al., 2005.

Isolates were screened for their ability to ferment 8 different carbohydrates (glucose, cellobiose, lactose, mannitol, maltose, ribose, sucrose, xylose) by using 96-well microtitre plates. For each test, overnight cultures were centrifuged for 10 min at 6 000 rpm and pelleted cells were washed and resuspended in MRS (without glucose) containing bromocresol purple as the pH indicator. Forty µl of filter sterilized (0.22 µm, Millipore) 10% sugar solutions and 60 µl of suspended cells were pipetted to each well. After 24 h incubation at 37°C, the plates were examined and the color change from purple to yellow indicated positive result for sugar fermentation. Glucose fermentation is considered as positive control, and samples without sugar were evaluated as negative control. (Bulut et al., 2005).

Genetic Identification

Four selected representative strains genetically were identified. Genomic DNA was obtained by using DNeasy Blood & Tissue Kit (Qiagen, Germany) and 16S rRNA genes were amplified by universal primers (Lane, 1991; Rudi et al., 1997). PCR was performed in 25-µl reaction mixture containing 200 ng template DNA, 12,5 µL 2X iProof HF Master Mix (BioRad, USA), 20 pmol/µL, for forward primer and 20 pmol/µL reverse primer in 0.2-µl tubes using a PCR System TC-300 (Techne, Cambridge, United Kingdom) under the following conditions: initial heat activation (5 min at 94°C), denaturation (30 cycles of 30 sec at 94°C), annealing (30 sec at 50°C), extension (45 sec at 72°C) and final extension (10 min at 72°C). Amplified genes were sequenced by using Bigdye Cycle Sequencing Kit v3.1 for each primer with ABI 3130XL Genetic Analyzer (RefGen, Ankara, Turkey). Aligned sequence was used for homology search by the Basic Local Alignment Search Tool (BLAST) software algorithm at National Center for Biotechnology Information (NCBI). The phylogenetic tree of the 16S rRNA

gene sequences were constructed with Geneious version 9 beta using neighbor-joining method.

Conjugated linoleic acid (CLA) production abilities

CLA concentrations were quantified by UV-spectrophotometric method (Terán et al., 2015; Torlak et al., 2016). Firstly, activated cultures were inoculated (5% v/v) to the MRS medium which contains 0.1 mg/mL Linoleic acid (LA; 99% purity; 0.902 g/mL density; Sigma-Aldrich) and incubated at 37°C for 24 h. The LA was previously in 30 mg/mL stock solution with 2% (v/v) Tween 80 then it was sterilized by filtration using 0.45 µm Minisart filter and stored at -20°C until use. After incubation, cells were separated by at 20,800 g for 5 min at 4°C, and lipid extraction was performed for their supernatants. They were mixed with 2 mL isopropanol and allowed to stand for 3 min. Then, 1.5 mL of hexane was added and vortexed for 3 min. Then, hexane layer was removed by centrifugation. In the experiments, control (MRS only without bacteria) is also included and CLA concentration was determined by measuring the absorbance at 233 nm. The calibration curve was obtained by using standard of cis-9, trans-11 CLA isomer (Sigma) was used to quantification of CLA in samples.

RESULTS AND DISCUSSIONS

Chemical Analysis

Acid levels of samples are important property and give information on expected microbial groups. pH values were between 3.13 and 5.46 with average 4.32 (Table 2).

Most of the samples have lower pH than 5.0. In general, pH values were similar both in the olive pulp and in the packing brine, except first sample. For Sample O1, the pH value of the pulp was lower (3.96) than brine (5.46) and its brine has the highest pH among the samples. The raising of pH of this sample can be explained by microbiological changes in the packing brine and Pereira et al. (2008) reported similar observations. For Sample O1, the pH value of the pulp was lower (3.96) than brine (5.46) and its brine has the highest pH among the samples. This can indicate that fermentation has just initiated. Variable titration acidity fractions were obtained (0.03-0.99). During fermentation, different microbial groups can produce organic acids and it cause decrease in pH value. Change in pH value also affects the dominant microbial flora type and organoleptic properties of the products.

Microbial Analysis

For traditionally produced samples, results reveal that diversity of microflora as presented in Table 2. The counts of aerobic mesophilic bacteria are between 4.5 and 7.2 log cfu/g. The range of fungal organisms and LAB counts were between (4.5–6.2 log cfu/g) and (3.8–7.4 log cfu/g)

Table 2. Table olive samples and their chemical and microbial count analyses

Sample	Sample information	pH	Titration acidity	Total aerobic mesophilic bacteria	LAB (log cfu/g)	Enterobacteria ceae (log cfu/g)	Staphylococci (log cfu/g)	Yeast and Molds (log cfu/g)
O1	Green olive	3.96	0.88	4.5	3.8	-	-	4.5
O1 brine		5.46	0.03	6.3	6.7	-	-	6.1
O2	Black olive	4.00	0.98	6.9	7.4	-	-	6.2
O2 brine		3.99	0.99	7.5	7.3	-	-	6.0
O3	Black olive(with oil)	3.85	0.79	7.2	6.8	6.1	-	5.5
O4	Black olive(Sele type)	4.29	0.99	5.7	5.5	-	-	6.5
O5	Black olive (Sele type)	4.97	0.72	5.7	5.7	6.5	-	6.2
O6	Green olive	4.25	0.55	6.6	6.6	-	-	6.2
O6 brine		4.43	0.1	6.3	6.1	-	-	5.9
O7	Black olive	4.49	0.83	6.0	6.1	-	-	5.7
O7 brine		4.89	0.05	5.4	5.8	-	-	6.3
O8	Dark green olive	4.81	0.46	6.0	6.5	-	-	5.8
O8 brine		5.04	0.03	6.2	6.0	-	-	6.3
O9	Green olive	3.31	2.22	-	-	-	-	-
O9 brine		3.13	1.74	-	-	-	-	-

respectively. Countable Enterobacteriaceae number is detected for only limited samples (O3 and O5). Heperkan (2013) also reported that Enterobacteriaceae species can be present in table olives at the beginning of the fermentation especially and they were disappeared during fermentation. In sample 9 (O9), no viable organism was detected and microbial inactivation can be considered for its preparation.

Isolation and Characterization of Lactobacilli

Among 233 isolated strains, 140 of them were purified. During streaking, some colonies have found to be similar to the yeast colony morphology then they were checked under microscope and they were not included in this work. Retry part of organisms were identified as *Lactobacillus*. Forty one representative isolates were selected and physiological tests indicated that most of the isolated strains have homofermentative property and mesophilic character (Table 3 and Table 4). Only one isolated strain (AK18 from O3 sample) showed gas formation from glucose and this result indicated heterofermentative property. Homofermentative property can be preferred for development of starters for table olive fermentations. Among isolated strains, only 2 of them hydrolyzed arginine and produced ammonia. All of the isolates grew well at 4% NaCl concentration. Most of them exhibited growth in 6.5% NaCl (34/41). None of the isolated strain showed good growth at 10% NaCl, only weak growths were observed for limited number of isolates. Good growth ability at high salt concentrations is one of the important criteria for selection suitable strains for table olive production. Most of the

isolates showed growth at 15°C but only a few amount of isolates exhibited growth at 45°C. Most of the isolates were found to be mesophilic nature and this property can also be desired for starter developments.

Seventeen different profiles are obtained by sugar fermentation tests (Table 3). All of the isolates fermented glucose, cellobiose, maltose, mannitol and sucrose and produced acid. Most of the isolates did not ferment xylose (28/41). Lactose fermentation and ribose fermentation found to be variable among the isolates.

Genetic Identification

The sequences of the selected strains AK15, AK19, AK25 and AK2 were found identical to *Lb. pentosus* sequence with 99% identity, *Lb. plantarum* sequence with 98% identity, *Lb. plantarum* sequence with 97% identity, *Lb. plantarum* sequence with 98% identity, respectively. The GenBank accession numbers for AK15, AK19, AK25 and AK29 are MG735259, MG735333, MG735356 and MG735359, respectively. Although various phenotypic profiles were obtained by sugar fermentation profile, all representatives were identified as *Lb. plantarum* and *Lb. pentosus* by 16S rRNA sequencing. These atypical profiles may be due to the different strains of same species. Phylogenetic analysis reveals that 4 main clusters were obtained (Fig1.). Cluster I was subdivided into two groups and isolated strains were clustered together in the first group that include *Lb. plantarum* and *Lb. pentosus* reference strains. Other reference strains present in the different branches of the phylogenetic tree.

Table 3. Grouping by fermentation sugar profile

Sugar Fermentation Profile	Codes for isolated strains	Cellobiose	Lactose	Mannitol	Maltose	Ribose	Sucrose	Xylose
Group 1	AK1,AK2,AK4, AK5,AK8,AK10	+	- (±)	+	+	-	+	-
Group 2	AK3	+	+(±)	+	+	-(±)	+	-
Group 3	AK9	+		+	+	-	+	-
Group 4	AK11,AK12, AK14,AK15	+	+	+	+	+	+	-(±)
Group 5	AK13	+	+	+	+	-(±)	+	-
Group 6	AK16	+	-	+	+	+	+	-
Group 7	AK17	+	+(±)	+	+	+	+	-
Group 8	AK19,AK20,AK21,AK22,AK23	+	+	+	+	+(±)	+	+(±)
Group 9	AK25,AK29,AK30,AK33, AK34,AK35,AK36	+	+	+	+	+(±)	+	-
Group 10	AK31,AK32	+	+(±)	+	+	+(±)	+	-
Group 11	AK37,AK42,*REF 1,**REF 2	+	+	+	+	+	+	-
Group 12	AK43,AK47	+	+	+	+	+	+	+
Group 13	AK39	+		+	+	+	+	+
Group 14	AK40	+	+(±)	+	+	(±)	+	-
Group 15	AK41	+	+	+	+	+	+	+
Group 16	AK48,AK50,AK51	+	+	+	+	+	+	+(±)
Group 17	AK45	+	-	+	+	-	+	-

+ (strong positive results), +(±) (weak positive), - (±) (weak negative), -(strong negative),*REF 1: *Lb. plantarum* NRRLB 4496; **REF2: *Lb. casei* NRRLB 441

Conjugated linoleic acid (CLA) production abilities

Those sequenced strains (AK 15, AK19, AK25 and AK29) produced variable amount of CLA in MRS medium 12(±2),15(±3),13(±1),20(±2) µg/mL respectively. CLA production ability is species and strain specific property and selection of best CLA producers can be important for further applications. Those strains could have potential to transform linoleic acid naturally found in table olive fermentation into CLA therefore this property can be evaluated for developing new starters with well-defined desirable health promoting properties.

CONCLUSIONS

Table olive fermentation has gained scientific attraction due to the health promoting properties and native lactic acid bacteria flora in recent years. According to results of this work, natural table olives contained significant amount LAB in their both pulps and brines. Although various phenotypes were found, mainly *Lb. plantarum* and *Lb. pentosus* were identified by genotyping tools. Among

isolated strains, *Lb. pentosus* AK15 and *Lb. plantarum* AK19 strains can be proposed for table olive fermentations due to their good growth abilities at 15°C and 6.5% and 10% salt concentrations. Additionally, CLA production abilities of those strains can be evaluated for production of health promoting fatty acid in table olive fermentation. Although there is no common application of starter LAB for industrial table olive production in nowadays, these strains with desired technological properties (homofermentative property, growth ability at low temperature and growth at high salt concentrations, CLA production ability) can be suggested for more controlled table olive fermentations in the future.

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Table 4. Results for physiological and biochemical tests

No	Code	Source	Gas from Glucose	Growth at 15 °C	Growth at 45 °C	Growth at 4% salt	Growth at 6.5% salt	Growth at 10% salt	Arginine hydrolysis	Sugar Ferm. Profile
1	AK1	O1	-	+	(±)	+	+	+(±)	-	Group 1
2	AK2	O1	-	+	+	+	+	+(±)	-	Group 1
3	AK3	O1	-	+	(±)	+	+	+(±)	-	Group 1
4	AK4	O1	-	+	(±)	-	+	+(±)	-	Group 1
5	AK5	O1	-	+	(±)	+	+	+(±)	-	Group 1
6	AK8	O1-brine	-	+	(±)	+	+	+(±)	-	Group 1
7	AK9	O1-brine	-	+	(±)	+	+	+(±)	-	Group 3
8	AK10	O1-brine	-	+	+	+	+	+(±)	-	Group 1
9	AK11	O1-brine	-	-(±)	(±)	+	+	+(±)	-	Group 4
10	AK12	O2	-	+	(±)	+	+	+(±)	-	Group 4
11	AK13	O2	-	+	-	+	+	-	-	Group 5
12	AK14	O2	-	+	(±)	+	-	+(±)	-	Group 4
13	AK15	O2-brine	-	+	-(±)	+	+	+(±)	-	Group 4
14	AK16	O2-brine	-	+	-(±)	+	+	+(±)	-	Group 6
15	AK17	O2-brine	-	+	-(±)	+	+	+(±)	-	Group 7
16	AK18	O3-brine	+	+	-	+	+	+(±)	+	N.D.
17	AK19	O3	-	+	-	+	+	+(±)	-	Group 8
18	AK20	O3	-	+	+	+	+	+(±)	-	Group 8
19	AK21	O4	-	-	-(±)	+	+	+(±)	-	Group 8
20	AK22	O4	-	-	-(±)	+	+	-	-	Group 8
21	AK23	O4	-	-	-(±)	+	+	-	-	Group 8
22	AK25	O5	-	-	-(±)	+	-	-	-	Group 9
23	AK29	O6	-	+	-(±)	+	-	-	+	Group 9
24	AK30	O6	-	+	-(±)	+	-	-	-	Group 9
25	AK31	O6	-	+	-(±)	+	-	-	-	Group 10
26	AK32	O6-brine	-	+	-(±)	+	-	-	-	Group 10
27	AK33	O6-brine	-	+	-(±)	+	-	-	-	Group 9
28	AK34	O6-brine	-	+	-(±)	+	-	-	-	Group 9
29	AK35	O6-brine	-	+	-(±)	+	-	-	-	Group 9
30	AK36	O7	-	+	-(±)	+	-	-	-	Group 9
31	AK37	O7	-	+	-(±)	+	-	-	-	Group 11
32	AK39	O7-brine	-	+	-(±)	+	+	-	-	Group 13
33	AK40	O7-brine	-	+	-(±)	+	-	-	-	Group 14
34	AK41	O7-brine	-	+	-(±)	+	-	-	-	Group 15
35	AK42	O7-brine	-	+	-(±)	+	-	-	-	Group 11
36	AK43	O8	-	+	-(±)	+	-	-	-	Group 12
37	AK47	O8-brine	-	+	-(±)	+	+-	-	-	Group 12
38	AK48	O8-brine	-	+	-(±)	+	-	-	-	Group 16
39	AK50	O8-brine	-	+	-(±)	+	-	-	-	Group 16
40	AK51	O4	-	+	-(±)	+	-	-	-	Group 16
41	AK45	O8	-	+	-(±)	+	-	-	+	Group 17

+ (strong positive results), +(±) (weak positive), - (±) (weak negative), -(strong negative)

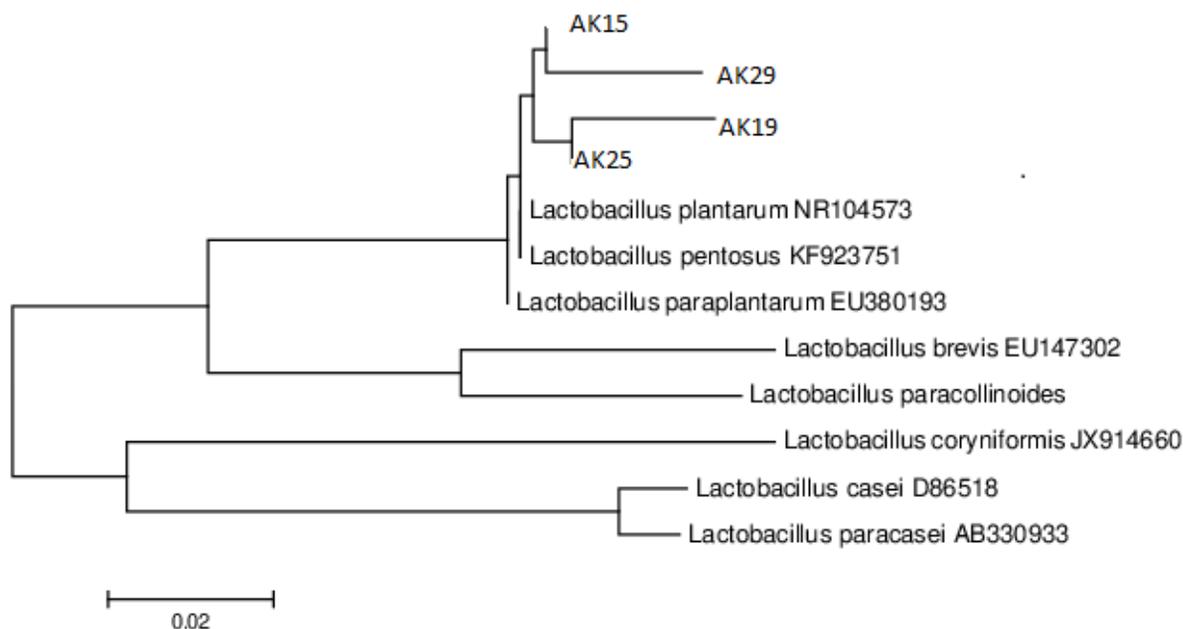


Figure 1. *Lactobacillus* phylogenetic tree based on whole 16S rRNA gene sequences of *Lactobacillus* spp. (constructed using ClustalW on Mobyl portal)

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