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ISOLATION AND IDENTIFICATION OF LACTIC ACID BACTERIA IN WINTER SALAD (LOCAL PICKLE) DURING FERMENTATION USING 16S rRNA GENE SEQUENCE ANALYSIS

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ABSTRACT

Winter salad is a kind of pickle made of different vegetables and is consumed specially in Khorasan Province. The spontaneous fermentation process is mainly carried out by lactic acid bacteria (LAB). The aim of this study was to isolate and identify the LAB species involved in winter salad fermentation at different fermentation time intervals to introduce the native strains. In order to classify selected isolates that seemed LAB, according to preliminary experiments, biochemical tests were performed. Forty-five selected isolate genotypically identified based on 16S rRNA gene sequencing. The results show that the analysis with 16s rRNA gene sequencing could successfully identify eight species, including *Pediococcus* (51.1%), *Lactobacillus* (26.7%), *Weissella* (11.1%), *Enterococcus* (6.7%) and *Leuconostoc* (4.4%). Although *Weissella cibaria* and *Lactobacillus plantarum* were the predominant LAB in the early periods of winter salad fermentation, *Pediococcus pentosaceus* was the predominant LAB at the later periods.

PRACTICAL APPLICATIONS

Fermented vegetables are usually used as seasoning in Iran. To our knowledge, this is the first study to examine fermented vegetables lactic acid flora using molecular methods, in Iran. Indigenous starter bacteria are normally considered as genetic resources, and strains obtained from this study could be used in the industry and some of them may be considered as probiotic strains.

INTRODUCTION

Lactic acid bacteria (LAB) constitute a group of gram-positive, nonsporing, nonrespiring cocci or rods, which produce lactic acid as the major end product during the fermentation of carbohydrates. From food technology point of view, the following genera are considered the principal LAB: *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Axelsson 2004). The spontaneous fermentation of food has been utilized for more than 5,000 years by mankind. It involves different products such as dairy products such as cheese, yoghurt and kefir; meat products such as salami or ham; and vegetable products such as sauerkraut or olives (Geisen and Holzapfel 1996).

Fermented vegetable foods are culturally and economically important, and their suitability in remote developing areas has made them an important component of the millions of people's diet. Therefore, they have a worldwide interest because of their sensory properties, their extended shelf life and as carriers of probiotic bacteria such as LAB (Abriouel *et al.* 2012). Most of the LAB involved in spontaneous fermentation of vegetables are *Lactobacillus* spp. (*Lactobacillus plantarum*, *La. fermentum*), *Leuconostoc* spp. (*Le. mesenteroides*), *Pediococcus* spp. (*P. pentosaceus*,

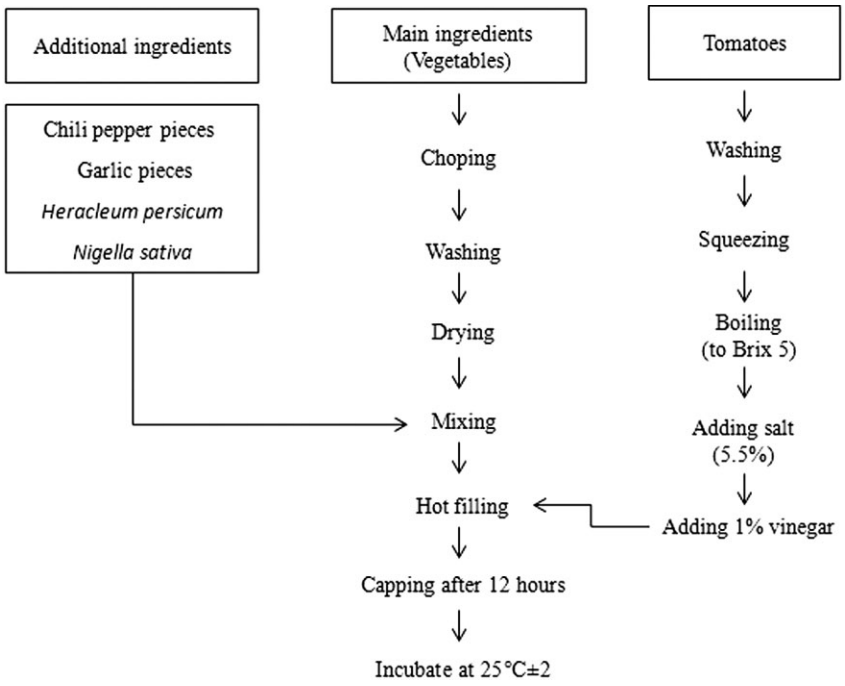

FIG. 1. FLOW DIAGRAM FOR WINTER SALAD PRODUCTION

P. acidilactici) and *Weissella* spp. (*W. cibaria*, *W. koreensis*) (Kim and Chun 2004; Perez Pulido *et al.* 2005; Tamang *et al.* 2005; Chen *et al.* 2010; Emerenini *et al.* 2013).

Like many Middle Eastern countries, Iran has a long tradition in producing several types of fermented vegetable products that are frequently used as seasoning in Iran. In fact, winter salad is a kind of pickle made of different vegetables and is consumed specially in Khorasan Province. An overview of the production of winter salad is shown in Fig. 1.

The polymerase chain reaction (PCR)-based methods are useful to identifying bacteria as a complementary or alternative tool to phenotypical methods. Over the years, identification methodologies use primers that target different sequences, such as the 16S ribosomal RNA encoding gene, the 16S-23S rRNA intergenic spacer region and the 23S rRNA encoding (Mohania *et al.* 2008). Today, the bacterial 16S ribosomal RNA operon, encompassing 16S rRNA and 23S rRNA genes, is the most frequently used as a molecular marker in microbial ecology because of a number of reasons including: (1) its evolutionary and phylogenetic properties, reflected by the presence of both variable and highly conserved sequence domains; (2) its high discriminatory potential; and (3) the extensive availability of sequences in public databases (Juste *et al.* 2008). Several studies have been carried out on the LAB composition of various vegetables, using 16S rRNA gene sequence analysis and phenotypic identification (Tamang *et al.* 2007; Park *et al.* 2010; Patil *et al.* 2010; Pang *et al.* 2011).

In this study, the profile of microbiological biodiversity and physicochemical changes of winter salad samples during the fermentation interval times (0, 7, 14, 21 and 28 days) were evaluated. Lactic acid flora of winter salad was studied by the classical culturing method and also by molecular analysis-based 16S rRNA gene sequence analysis.

MATERIALS AND METHODS

Production of Winter Salad

The vegetables (cabbage, carrot, pepper, garlic, cauliflower, Jerusalem artichoke and celery) used for the production of winter salad were purchased from local markets in Mashhad, Iran. According to the traditional production instruction of Winter salad the vegetables, were chopped and washed with fresh water and then were dried for 30 min. The vegetables were pooled and placed in 250 g jars; a few pieces of chili peppers and garlic with a little *Heracleum persicum* (golpar) and *Nigella sativa* seeds were also added to each jar. Then the jars were filled with tomato juice (Brix 5) and 5.5% (w/w) edible salt. In order to acidification, 1% of vinegar was added to tomato juice. Forty-five jars were produced and after 12 h the jar lids were tightly closed. Samples were stored at room temperature 25 ± 2C.

Sampling

As ripening period of winter salad is 3 weeks, the sampling was performed in five 7-day intervals (day 0, day 7, day 14 and day 21) and day 28 as after ripening period. Sampling was performed from six jars per day and winter salad LAB

isolated and enumerated in Man Rogosa Sharpe agar aerobically and anaerobically in mentioned time intervals. Ten grams of sample was homogenized in a Stomacher (400; Seward, Worthing, UK), with 90 mL of 0.85% w/v sterile physiological saline for 5 min and serially diluted (10^{-1} to 10^{-8}). One hundred microliters of these dilutions was pour plated in the MRS agar (Merck, Darmstadt, Germany). LAB were detected and isolated on MRS agar after incubation under both aerobic and anaerobic conditions in an anaerobic gas pack system (Merck) at 25, 37 and 45C for 48–72 h. Distribution of mesophilic and thermophilic LAB in the samples was investigated using three different incubation temperatures: 45C for thermophilic LAB and 25 and 37C for mesophilic ones in three replicates for each temperature. Purity of the isolates was checked by streaking again and subculturing on fresh agar plates of the isolation media. Purified strains of LAB were preserved in MRS broth using 15% v/v glycerol at −80C (Tamang *et al.* 2005).

pH and Titratable Acidity

Ten grams of each sample was blended with 20 mL of distilled water, pH was determined directly using a digital pH meter (Metrohm 744 pH meter, Herisau, Switzerland). Titratable acidity was determined by titrating brine up to pH 8.2 with 0.1 N NaOH and expressed as grams of lactic acid per 100 mL brine (Panagou *et al.* 2008).

Morphological, Physiological and Biochemical Tests

Overnight cultures of LAB isolates were Gram stained, and cell morphology of all strains of LAB was determined in a phase contrast microscope (Olympus BX41-TF, Tokyo, Japan). Catalase test was carried out by adding few drops of freshly prepared 3% hydrogen peroxide to each plate containing 18-hour-old culture of each isolate. In order to classify 136 selected isolates that according to preliminary experiments seemed to be LAB, biochemical tests include carbon dioxide production from glucose, growth at 10 and 45C, growth at 6.5% NaCl, growth at pH = 4.4 and pH = 9.6, and hydrolysis of arginine was performed (Schillinger and Lucke 1987; Dykes *et al.* 1994). Carbohydrate fermentation tests were performed in Phenol Red Broth base (Quelab, Dandurand, Montreal, QC, Canada) with 1% mannitol, sorbitol, maltose, fructose, melibiose, sucrose, galactose, raffinose, glucose and lactose (Merck) (Faklam and Collins 1998).

DNA Extraction

Fifty isolates were selected for molecular identification. Total genomic DNA was extracted from 0.1 mL sample of overnight cultures grown in MRS broth, with a Genomic DNA isolation kit (Dena Zist Asia, Mashhad, Iran).

PCR Amplification of 16S rRNA Gene and Electrophoresis

The universal primer pair consisted of the forward (5′ GAG AGT TTG ATC CTG GCT CAG 3′) and reverse (5′ GAA AGG AGG TGA TCC AGC CG 3′), which were used for amplification of 16S rDNA that were obtained from Fermentase (Burlington, ON, Canada). Deoxyribonucleic acid amplification of the (~1,500 bases) fragment was carried out in a 20 μL reaction mixture containing 2.5 μL of 10 × PCR buffer, 1 μL deoxynucleoside triphosphate mixture (10 mM), 1.2 μL of MgCl2 (25 mM), 1 μL of each primer (100 pmol/μL), 3 μL of DNA (10 ng) and 0.3 μL of Taq DNA polymerase (5 U/μL). All PCR chemicals were obtained from Cinnagen (Tehran, Iran). Amplification was performed in a SensoQuest Labcycler (D-37085, Goettingen, Germany). The PCR conditions were as follows: initial denaturation of DNA for 5 min at 95C, then 30 cycles of denaturation of DNA for 1 min at 94C, annealing for 1 min at 53C, extension for 2 min at 72C and final incubation for 5 min at 72C. The PCR products were analyzed by electrophoresis on a 1.2% agarose gel stained with Green viewer (Fermentase) in 1X TBE buffer at 90 V for 45 min. The sizes of DNA fragments were estimated using a standard 1 kb DNA ladder (Fermentase), and the gels were documented using the SSM-930 Vilber Lourmat gel documentation imaging (Eberhardzell, Deutschland) (Taheri *et al.* 2009). Then the PCR products were sent to Macrogen (Seoul, Korea) for purifying and sequencing. To determine the closest known relatives of the partial 16S rRNA gene sequences obtained, identification was performed in public data libraries (GenBank) using the Basic Local Alignment Search Tool (BLAST) (Park *et al.* 2010).

Phylogenetic Analysis

The sequences of 16S rRNA gene were edited using the Chromas Lite 2.1 (Technolysum, South Brisbane, Australia), CLC Main Workbench 5.5 program (QIAGEN, Boston, MA), Mega version 6.05 (MEGA, Tempe, AZ), SPSS version 16.0 (WinWrap, Nikoski, AK). The phylogenetic trees were constructed by neighbor-joining statistical method. *Escherichia coli* ECSC1 was used as an outgroup. In order to determine the stability of our phylogenetic tree, the sequence data were sampled 1,000 times for bootstrap analysis using Mega version 6.05 with 50% cutoff (Chao *et al.* 2007; Park *et al.* 2010).

Statistical Analysis

The data of counting LAB, pH and acidity were analyzed using SPSS version 16.0. A single-factor analysis of variance

TABLE 1. CHANGES IN PH, ACIDITY AND LACTIC ACID BACTERIA COUNTS OF WINTER SALAD ± STANDARD DEVIATION (SD) DURING RIPENING PERIODS

			Log cfu/g					
			Aerobic			Anaerobic		
Day	pH	Acidity (%)	25C	37C	45C	25C	37C	45C
0	4.19 ± 0.04^{a}	0.571 ± 0.012^{a}	3.73 ± 0.04^{a}	4.07 ± 0.02^{a}	2.36 ± 0.07^{a}	3.96 ± 0.08^{a}	4.18 ± 0.14^{a}	2.50 ± 0.15^{a}
7	3.91 ± 0.07^{b}	0.898 ± 0.061^{b}	8.32 ± 0.07^{b}	8.56 ± 0.15^{b}	7.86 ± 0.04^{b}	8.45 ± 0.12^{b}	8.49 ± 0.09^{b}	7.59 ± 0.07^{b}
14	3.87 ± 0.05^{b}	0.930 ± 0.054^{b}	8.91 ± 0.09^{c}	9.31 ± 0.03^{c}	8.57 ± 0.02^{c}	9.20 ± 0.05^{c}	9.36 ± 0.02^{c}	8.56 ± 0.02^{c}
21	3.80 ± 0.05^{b}	1.084 ± 0.022^{c}	10.68 ± 0.01^{d}	10.88 ± 0.06^{d}	9.87 ± 0.11^{d}	10.81 ± 0.03^{d}	10.85 ± 0.03^{d}	10.08 ± 0.19^{d}
28	3.62 ± 0.02^{c}	1.396 ± 0.034^{d}	8.87 ± 0.02^{c}	9.10 ± 0.14^{c}	8.60 ± 0.09^{c}	9.21 ± 0.06^{c}	9.50 ± 0.12^{c}	8.73 ± 0.08^{c}

cfu, colony-forming unit.

was used to calculate the standard error of the mean of the treatments. For all tests, differences were considered significant at $P < 0.01$. All experiments were performed in duplicate. Duncan multiple range tests with a confidence interval of 99% were used to compare the means.

RESULTS AND DISCUSSION

LAB Counts, pH and Acidity during Winter Salad Fermentation

Acidity, pH and LAB counts of winter salad are shown in Table 1. The initial count of LAB at the start of fermentation (day 0), immediately after production, was low. In 25C, the LAB numbers exceed 4.15 log colony-forming unit (cfu)/g in 37C, 3.84 log cfu/g in 45C and 2.43 log cfu/g. After 7 days LAB counts had grown at all three temperatures and were almost doubled. During fermentation on days 14 and 21, this growth continued until near 10.5 log cfu/g. Ripening period of winter salad is 3 weeks and usually after this period, the product could be consumed. On day 28 after ripening period, LAB counts showed a decreasing trend probably reflecting beginning nutrient shortage. Decline in LAB counts was seen and it was near 9 log cfu/g. As it is clear, various stages of fermentation had significant effect on LAB count grown on MRS agar medium except days 14 and 28 at all three temperatures ($P < 0.01$).

The initial pH of winter salad on day 0 was 4.19, and titratable acidity based on lactic acid was 0.571% due to tomato juice in formulation. On day 0 and day 28, pH was significantly different from the other days ($P < 0.01$). During the fermentation of products, continuous decline in pH and increase in acidity was observed consistently. It shows that increasing the fermentation time causes higher lactic acid content in the product until day 21. But the highest acidity and the lowest pH were on day 28. Decrease in pH and increase in acidity despite the reduction in the number of bacteria on day 28 may be due to the high potential of acidification of predominant LAB in this period. There was significant difference in acidity at various days except on days 7 and 14 ($P < 0.01$).

Identification

One hundred and thirty-six gram-positive, catalase-negative isolates were obtained from various periods during winter salad fermentation. To classify that the isolates of LAB strains were phenotypically characterized on the basis of cell morphology, gas production from glucose, arginine hydrolysis, growth at different temperatures and pH, the sugar fermentation patterns were determined (Table 2). All isolates based on physiological and biochemical characteristics were divided into 10 groups. Fifty isolates from 136 isolates were selected from these groups for genotypical characterization by 16S rRNA gene sequence analysis. The isolates were chosen that contains all ten groups and all various ripening stages. After comparing 16S rRNA gene sequences obtained, with available sequences in database using the BLAST, 45 isolates that had high similarity (97% and more) were selected (Table 3). The results of this study showed that the analysis with 16S rRNA gene sequencing could be successfully identified in eight species, including *P. pentosaceus* (51.1%), *La. plantarum* (20%), *W. cibaria* (11.1%), *La. fermentum* (6.7%), *Enterococcus faecium* (4.4%), *En. faecalis* (2.2%), *Leuconostoc citreum* (2.2%) and *Leuconostoc mesenteroides* subsp. *mesenteroides* (2.2%).

Isolate series A was obtained from winter salad immediately after production. In conclusion, *W. cibaria*, *Le. citreum*, *La. plantarum* and *P. pentosaceus* were detected in the raw materials where *W. cibaria* was the dominant species. *Weissella* species are widespread in the natural environment. *W. cibaria* has been found in fermented foods (cassava, sourdough and the Japanese alcoholic beverage shochu) (Chao *et al.* 2007). Also Emerenini *et al.* (2013) found it as dominant LAB from fresh fruits and vegetables in Nigeria.

Isolates series B and C include *P. pentosaceus* and *La. plantarum* obtained from day 7. After 2 weeks on day 14 (series D and E), the highest diversity of bacteria covering

TABLE 2. PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF THE LAB ISOLATED FROM WINTER SALAD

Group number	1	2	3	4	5	6	7	8	9	10
Number of strains	22	7	11	17	51	9	8	2	5	4
Growth at 10C	+	+	+	+	–	–	+	+	+	+
Growth at 45C	–	(5/2)	(8/3)	+	(6/45)	–	–	–	+	+
Growth at pH = 4.4	+	–	–	(6/11)	+	+	(2/6)	–	+	+
Growth at pH = 9.6	(3/19)	+	+	–	–	–	–	–	+	+
Growth at 6.5% NaCl	(20/2)	–	–	(7/10)	+	+	(1/7)	–	+	+
Arginine hydrolysis	–	+	+	+	+	+	–	–	+	+
CO_2 from glucose	–	+	+	+	–	–	+	+	–	–
Glucose	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	–	+	+	+	–	+
Galactose	+	+	+	–	+	+	–	+	+	+
Fructose	+	+	–	+	+	+	+	+	+	+
Lactose	–	+	+	–	+	–	–	+	+	+
Maltose	+	+	+	+	+	+	+	+	–	+
Sorbitol	+	–	–	–	–	–	–	–	–	–
Raffinose	+	(1/6)	–	(2/15)	–	–	–	–	+	–
Manitol	+	–	(6/5)	–	–	–	+	+	(4/1)	+
Mellibiose	+	+	+	–	–	–	–	+	–	–

+, all strains positive; –, all strains negative; (../..), number of positive/negative strains.
LAB, lactic acid bacteria.

six species, *La. plantarum*, *La. fermentum*, *P. pentosaceus*, *En. faecium*, *Le. mesenteroides* subsp. *mesenteroides* and *W. cibaria*, was obtained and *La. plantarum* was dominant in this period. Three species, *P. pentosaceus*, *La. plantarum* and *En. faecium*, were LAB on day 21 (series F and G). *P. pentosaceus* was predominant LAB at this period, and this domination continued on day 28 so that six strains of seven identified strains on this day (series H and I) belonged to this species, and the other was *En. faecalis*.

Lactobacillus and *Leuconostoc* spp. and in the low rate *Pediococcus* spp. are found on plants (Stiles and Holzapfel 1997); in this study, these genera with *Weissella* were found on raw materials. At first, heterofermentative strains such as *Weissella* and *Leuconostoc* strains began the fermentation with gas production. Absence of *Leuconostoc* in the other interval times except for one case may be due to decreased pH because these species are nonacidophilic and prefer an initial medium pH of 6.5 (Schleifer 2009).

During the fermentation process, heterofermentative strains were gradually replaced by homofermentative strains. The closest product to winter salad with identified LAB is Kimchi, a Korean fermented vegetable food; LAB mostly found in Kimchi fermentation belong to genera *Weissella*, *Leuconostoc* and *Lactobacillus* and to a lesser extent *Pediococcus* and *Enterococcus* (Choi *et al*. 2002; Lee *et al*. 2002, 2005; Kim and Chun 2004; Cho *et al*. 2006; Park *et al*. 2010), but the interesting point in this study is that the *P. pentosaceus* with 51.1% was dominant among total LAB. A study on caper berries has obviously shown that in the early stages of fermentation, *Pediococccus* spp. was predominant but after that *Lactobacillus* spp. overcame)Perez Pulido *et al*. 2005). As mentioned in this study, contrary *Pediococccus* are overpowered on the other genera.

P. pentosaceus is involved in many plant fermentation, and during fermentation this product was ubiquitous, probably due to the high salt tolerance and its compatibility with acidic conditions (Daeschel *et al*. 1987; Schleifer 2009). Singh and Ramesh (2008) also found that *Lactobacillus* and *Pediococcus* were the dominant genera after 36 h of cucumber fermentation.

The isolates and related reference strains were used to construct the phylogenetic tree (Fig. 2). To facilitate, after sequences alignment, only one of the quite similar isolates was chosen to draw the phylogenetic tree. Tree mainly composed of four clusters and six subclusters including eight genera. Cluster I was the *Pediococcus* group that was composed of two subclusters: subcluster i *P. pentosaceus* and subcluster ii *La. plantarum*. Cluster II (subcluster iii) was *La. fermentum*. Cluster III (subcluster iv) was *Enterococcus* group. Cluster IV was *Leuconostoc* group that was composed of two subcluster including subcluster v, the *W. cibaria*, and subcluster vi, the group of *Leuconostoc*.

CONCLUSIONS

Results showed that, in early fermentation period, LAB attended as part of the vegetables microbial population. Twenty-one-day fermentation period brought about a reduction in pH, and an increase in the useful acid lactic microflor that lasted to the end. In the molecular determination phase, selected isolates were strains of LAB that are dominant species during the winter salad fermentation. This type of fermented

TABLE 3. IDENTIFICATION OF LACTIC ACID BACTERIA ISOLATES FROM WINTER SALAD

Code of isolate	Day of sampling	Closest strain in NCBI database	Accession number	Percentage similarity (%)
A5	0	*Weissella cibaria* II-I-59	NR_036924.1	100
A6	0	*W. cibaria* II-I-59	NR_036924.1	100
A12	0	*Leuconostoc citreum* KM20	NR_074694.1	99
A13	0	*W. cibaria* II-I-59	NR_036924.1	100
A25	0	*Lactobacillus plantarum* WCFS1	NR_075041.1	99
A34	0	*Pediococcus pentosaceus* ATCC 25745	NR_075052.1	97
A42	0	*Weissella cibaria* II-I-59	NR_036924.1	100
B11	7	*P. pentosaceus* ATCC 25745	NR_075052.1	99
B22	7	*P. pentosaceus* ATCC 25745	NR_075052.1	99
B23	7	*La. plantarum* PD412	AB854180.1	99
B73	7	*La. plantarum* WCFS1	NR_075041.1	99
C1	7	*P. pentosaceus* ATCC 25745	NR_075052.1	99
C2	7	*P. pentosaceus* ATCC 25745	NR_075052.1	100
C5	7	*P. pentosaceus* ATCC 25745	NR_075052.1	99
C14	7	*P. pentosaceus* ATCC 25745	NR_075052.1	99
C33	7	*P. pentosaceus* ATCC 25745	NR_075052.1	98
D2	14	*La. fermentum* IFO 3956	NR_075033.1	98
D7	14	*La. plantarum* WCFS1	NR_075041.1	99
D14	14	*La. fermentum* IFO 3956	NR_075033.1	98
D18	14	*P. pentosaceus* ATCC 25745	NR_075052.1	100
D40	14	*W. cibaria* II-I-59	NR_036924.1	100
D46	14	*Le. mesenteroides* subsp. *mesenteroides* ATCC 8293	NR_074957.1	97
D47	14	*La. plantarum* WCFS1	NR_075041.1	97
D60	14	*P. pentosaceus* ATCC 25745	NR_075052.1	99
E1	14	*La. plantarum* WCFS1	NR_075041.1	99
E3	14	*La. plantarum* WCFS1	NR_075041.1	99
E5	14	*Enterococcus faecium* Aus0004	NR_102790.1	97
E7	14	*La. fermentum* IFO 3956	NR_075033.1	98
F5	21	*La. plantarum* PD412	AB854180.1	99
F43	21	*P. pentosaceus* ATCC 25745	NR_075052.1	99
F45	21	*P. pentosaceus* ATCC 25745	NR_075052.1	99
G1	21	*P. pentosaceus* FMAC63	KF_060272.1	100
G25	21	*P. pentosaceus* SPA	KF_562066.1	97
G31	21	*P. pentosaceus* ATCC 25745	NR_075052.1	99
G37	21	*P. pentosaceus* ATCC 25745	NR_075052.1	100
G38	21	*P. pentosaceus* ATCC 25745	NR_075052.1	100
G39	21	*La. plantarum* WCFS1	NR_075041.1	99
G43	21	*En. faecium* Aus0004	NR_102790.1	97
H4	28	*P. pentosaceus* ATCC 25745	NR_075052.1	99
H8	28	*En. faecalis* V583	NR_074637.1	98
H11	28	*P. pentosaceus* ATCC 25745	NR_075052.1	99
H13	28	*P. pentosaceus* ATCC 25745	NR_075052.1	100
H17	28	*P. pentosaceus* ATCC 25745	NR_075052.1	99
I19	28	*P. pentosaceus* ATCC 25745	NR_075052.1	99
I21	28	*P. pentosaceus* wg8	JX_477168.1	100

NCBI, National Center for Biotechnology Information.

products has a wide microbial diversity that originates from natural microbiota presented in the raw vegetable and therefore production of a variety of acids and enzymes, which plays an important role in preservation and development of unique flavor and taste. The data obtained from this work provide useful frame for further studies on antimicrobial activity of LAB, their proteolytic and lipolytic activities, and ability to produce exopolysaccharides. Therefore, these strains could be used in the industry. However, the details of the microbes involved in the fermentation of this product are still unclear. Further investigations are needed to shed light on the microbial dynamics, and the analysis of more winter salad would contribute to a better understanding of the fermentation process.

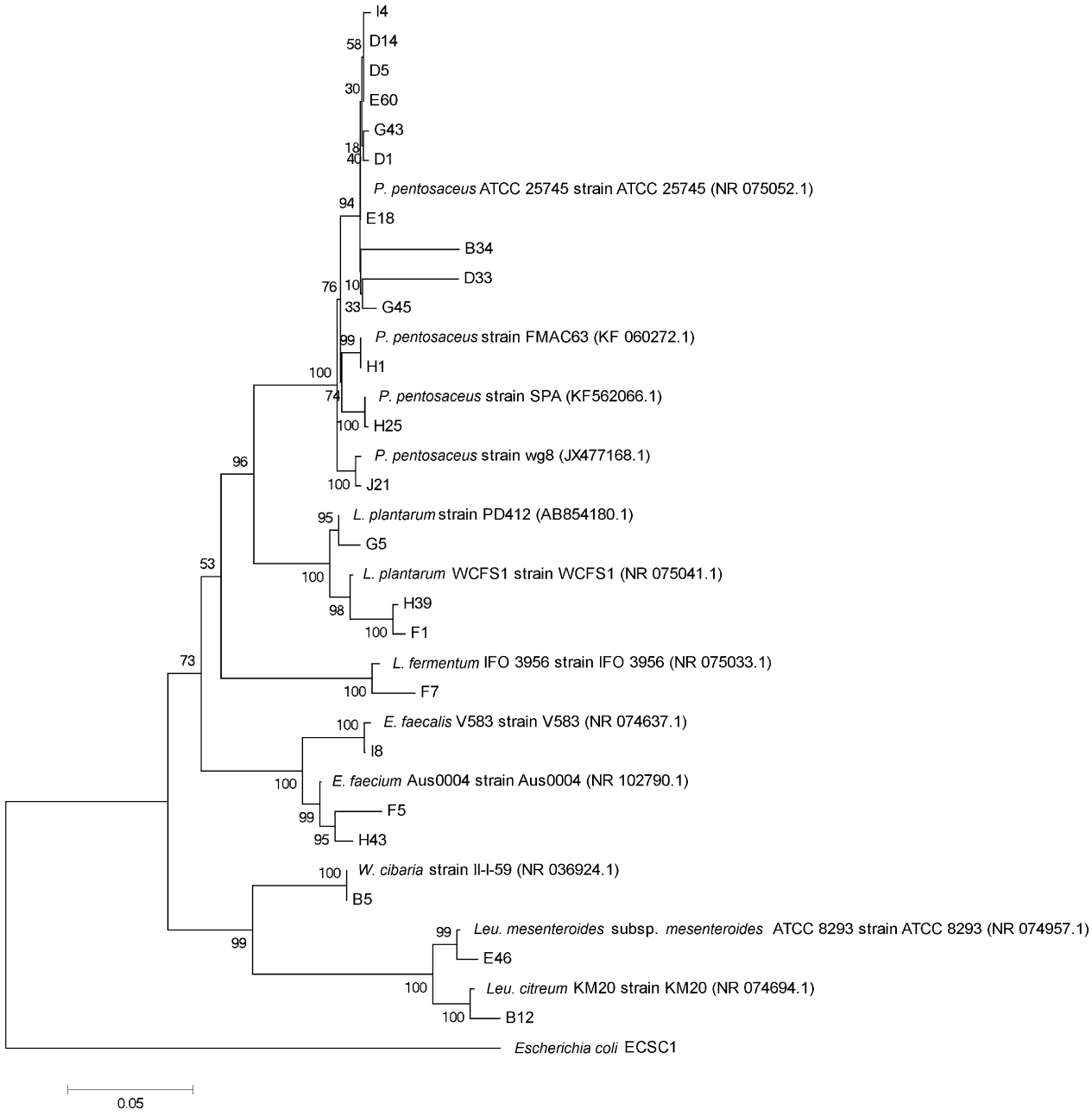

FIG. 2. PHYLOGENETIC TREE BASED ON 16S rDNA SEQUENCE ANALYSIS, SHOWING THE PHYLOGENETIC PLACEMENT OF STRAINS ISOLATED FROM WINTER SALAD

The tree was constructed by the neighbor-joining statistical method, and *Escherichia coli* was used as the outgroup. Bootstrap values for a total of 1,000 replicates are shown at the nodes of the tree. References of the type strains used for comparison are given.

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