Phenotypic and genotypic identification of lactic acid bacteria isolated from traditional pickles of the Çubuk region in Turkey

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Abstract A total of 152 lactic acid bacteria (LAB) were isolated from pickles produced in the Ankara-Çubuk region. These isolates were clustered into eight groups on the basis of their phenotypic characteristics including cell morphology, CO_2 production from glucose, growth at 10 and 45 °C, growth in 6.5 % NaCl, and growth at pH 9.6. API 50 CH carbohydrate fermentation test, 16S ribosomal RNA (rRNA) sequence analysis, and sodium dodecyl sulfate-acrylamide gel electrophoresis (SDS-PAGE) whole-cell protein profile analysis were also performed for precise identification of the isolates at the species level. Molecular identification revealed that the most prevalent LAB species involved in pickle fermentation were *Pediococcus ethanolidurans* (46 isolates, 30.3 %), *Lactobacillus brevis* (37 isolates, 24.3 %), *Lactobacillus plantarum* (37 isolates, 24.3 %), and *Lactobacillus buchneri* (15 isolates, 9.9 %). Other LAB were found in minor frequencies such as *Pediococcus parvulus* (8 isolates, 5.3 %), *Lactobacillus namurensis* (6 isolates, 3.9 %), *Lactobacillus diolivorans* (1 isolate, 0.7 %), *Lactobacillus parabrevis* (1 isolate, 0.7 %), and *Enterococcus casseliflavus* (1 isolate, 0.7 %). When results of phenotypic and genotypic identification methods were compared, differences in the species distribution of LAB associated with pickles were defined between the API and the 16S rRNA sequencing. The API 50 CHL test coincided with the 16S rRNA results in 71 out of the 152 tested isolates, indicating that API gave unreliable identification results. A clear correlation could not be found between the results of whole-cell SDS profiles and 16S rRNA sequencing. Therefore, molecular characterization by 16S rRNA sequencing was considered to be the most reliable method for identifying isolates. The results presented in this work provide insight in to the LAB population associated with traditional Çubuk pickles and constitute a LAB strain resource for further studies involving the development of starter cultures.

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Introduction

Pickle, a traditional fermented vegetable product, is very popular in the Çubuk region of Ankara, Turkey. Pickling has an important role in Turkish lifestyle. The Municipality of Çubuk has obtained a patent for "Çubuk Pickles." Pickle production relies typically on spontaneous fermentation by indigenous lactic acid bacteria (LAB) flora associated with vegetables, such as cucumber, cabbage, and green pepper. However, the sensory quality of the pickle products varies by the raw material and inherent microbial flora. Therefore, developing starter cultures for pickle production is crucial to standardize the fermentation and the quality of the final product. Although numerous lactic cultures are available for the dairy and meat industries, there are no commercial starters designed for pickle production. To our knowledge, no previous studies have been conducted to develop LAB starter cultures indigenous to Ankara-Çubuk pickles.

Phenotypic methods including morphological and physiological analysis, biochemical characterization by API 50 CHL

system and protein profiling by SDS-PAGE, have been widely used for the identification of LAB. However, the main drawbacks of phenotypic methods are poor reproducibility and low discriminatory power that do not allow precise identification at species level (Temmerman et al. 2004; Markiewicz et al. 2010). Although protein profiling is considered to be more reliable for LAB identification, it gives poor discrimination of *Lactobacillus* groups (Temmerman et al. 2004). Since *Lactobacillus* species are known as the predominant in pickle fermentation, a genome-based method is needed to be performed in this study. Moreover, a combined phenotypic and genotypic approach could be preferred for reliable characterization.

It is known that technological and probiotic properties are strain dependent, and so, reliable identification of LAB is of great importance before selecting starter cultures appropriate for industrial use (Sánchez et al. 2004). DNA sequencing of the 16S ribosomal RNA (rRNA) genes has been largely used for the identification of LAB (Balcázar et al. 2007; Pogačić et al. 2010; Moraes et al. 2013).

There are undoubtedly numerous published reports dealing with the identification of LAB isolated from traditional fermented vegetables in different countries/regions, such as China, Japan, and India (Tamang et al. 2005; Endo et al. 2008; Kingston et al. 2010; Yu et al. 2012). However, it is known that the distribution of microbial community in fermented vegetable products varies by region. In spite of being very popular in Turkey, very limited information is available on LAB associated with Çubuk pickles. Therefore, the objective of this study was to characterize and identify the predominant lactic acid bacteria present in pickles from the Ankara-Çubuk region. 16S rRNA sequencing coupled with SDS-PAGE of whole-cell proteins and biochemical characterization by API 50 CHL test could provide the precise identification of LAB involved in pickle fermentation. This study constitutes the first step in selecting LAB starter cultures indigenous to the Ankara-Çubuk region in order to preserve autochthonous strains and characteristic sensorial properties of traditional pickles.

Materials and methods

Isolation of LAB

Fifty three of pickle samples (prepared by using cucumber, cabbage, pepper, tomato, carrot, bean, and eggplant separately or in mixtures) were randomly collected from 18 individual household, local markets, and small factories in the Ankara-Çubuk region. Samples were collected in sterile bottles and kept at 4 °C after transported to the laboratory. The pH of brine samples was measured by a calibrated pH meter (Mettler Toledo, SevenEasy pH, China). Titratable acidity, expressed

as g lactic acid per 100 mL, was calculated by titrating the brine with 0.01 mol/L NaOH with 0.1 % (w/v) phenolphthalein as the indicator. The salt content of the brine, expressed as g NaCl per 100 mL, was determined by titration with 0.1 N AgNO₃ using 5 % (w/v) potassium dichromate (K_2CrO_4) as the indicator.

For colony counting and isolation of LAB, aliquots of brine samples were serially diluted with saline buffer containing 0.85 % (w/v) NaCl and plated on De Man-Rogosa-Sharpe (MRS) Agar (Merck, Germany) and M17 Agar (Merck, Germany). After incubation at 30 °C for 48–72 h, ten colonies showing different colony morphologies were picked from each plate of the highest dilution. Selected colonies were purified by using streak plate method and tested for cell morphology, Gram reaction, catalase, and oxidase activity. Gram-positive and catalase- and oxidase-negative cultures were considered to be presumptive LAB. The isolates were stored at −65 °C in 30 % (v/v) glycerol solution until identification analyses. Except where otherwise stated, the isolates were cultured at 30 °C for 48 h in MRS broth and/or MRS agar as basal media.

Phenotypic characterization of isolates

Phenotypic tests including cell morphology, CO_2 production from glucose, growth at different temperatures (10, 15, and 45 °C), growth in 6.5 % NaCl, growth at pH 9.6, and ammonia production from arginine were conducted followed by further identification using the commercial API 50 CH carbohydrate fermentation test. Cell morphology of the isolates was determined using a phase contrast microscope. CO_2 production from glucose was tested in MRS and M17 medium containing inverted Durham tubes. On the basis of the production of gas, cultures were considered as homofermentative/ heterofermentative (Hitchener et al. 1982; Randazzo et al. 2004). Growth at different temperatures (10, 15, 45 °C) was examined after 72 h of incubation. Growth in 6.5 % NaCl was observed after 7 days of incubation at 30 °C in MRS broth containing 6.5 % NaCl as described by Chao et al. (2009). Growth at pH 9.6 was observed after 2 days of incubation at 30 °C in MRS broth which pH was adjusted to 9.6. Production of NH₃ from arginine was determined according to Şimşek et al. (2006). Carbohydrate fermentation patterns were determined using API 50 CHL test strips according to manufacturer's instructions (bioMérieux, France).

Genotypic characterization of isolates

SDS-PAGE whole-cell protein profile analysis

Whole-cell proteins were extracted according to De Angelis et al. 2001, Kizerwetter-Swida and Binek 2005, and Kim and Adachi 2007, and subsequently, sodium dodecyl sulfate

(SDS)-acrylamide gel electrophoresis (PAGE) of the proteins was performed as described by Laemmli (1970). For the extraction of whole-cell proteins, cultures were incubated in MRS broth at 30 °C for 24–48 h. Cells were harvested and washed with 10 mL of distilled water by centrifugation at 4100g for 10 min. Washed cell pellets were resuspended in 100 μL lysozyme solution (BioChemica AppliChem GmbH, Germany) and incubated at 37 °C for 1 h vortexing every 10 min. Lysozyme-treated cells were then frozen at −20 °C and sonicated (VC 130, Sonics Vibra-Cell, USA) at 1000 amplitude for 1–2 min. After sonication, samples were mixed with 500 μL SDS-PAGE sample buffer (3.8 mL of distilled water, 1 mL of 0.5 mol/L Tris-HCl at pH 6.8, 0.8 mL of glycerol, 1.6 mL of 10 % (w/v) SDS, 0.8 mL of β-mercaptoethanol, 0.4 mL of 0.05 % (w/v) bromophenol blue), incubated at 95 °C for 5 min, and centrifuged at 14000g for 10 min. The supernatants were analyzed by SDS-PAGE with a vertical Mini-PROTEAN System (Bio-Rad Laboratories, USA). Briefly, 20 μL of supernatant samples were loaded onto 4–12 % (respective concentrations of stacking and separation gel) acrylamide gradient gels. Electrophoresis was performed using a power supply (EV231, Consort, Belgium) operated at 70 V through the stacking gel and 100 V through the separation gel for 2–3 h. The proteins were visualized by staining with Coomassie Brilliant blue G 250 dye solution (0.1 % (w/v) Coomassie Brilliant blue G 250 (Merck, Germany) 10 % (v/v) acetic acid, 50 % distilled water, 40 % ethanol) and were destained with the same solution without the addition of Coomassie Brilliant blue G 250. A standard protein solution (SM0661, Fermentas, EU), containing 14 proteins ranging in size from 10 kDa to 200 kDa, was used as a molar mass marker. Densitometry of SDS-PAGE gels was performed using Gel Logic 200 Imaging System (Kodak, USA). The similarity between protein band patterns of individual strains was estimated using the Dice coefficient, and dendrograms were derived from the similarity matrix by Unweighted Pair Group Method Using Average Linkage (UPGMA) cluster analysis that were performed using the statistiXL Version 1.8 software package.

16S rRNA gene sequences analysis

DNA extraction Genomic DNA was isolated using a QIAGEN DNeasy Blood & Tissue Kit (Germany) in accordance with manufacturer's instructions.

Amplification and sequencing of the 16S rRNA Amplification of bacterial 16S rRNA regions was carried out by using F27 (AGAGTTTGATCCTGGCTCAG) and R1492 (TACGGYTACCTTGTTACGACTT) primers. The reaction mixture consisted of 100 ng template DNA, 1× buffer, 25 mM MgCl, 0.2 mM dNTP, 0.4 pmol of each primer, and 1.25 U of Taq polymerase in a final volume of 50 μL (Lane 1991). PCR

amplifying protocol was as follows: a primary denaturation cycle at 94 °C for 5 min, then 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and elongation at 72 °C for 1 min.

Sequencing of the PCR fragments was conducted by a biotechnology company (Refgen Biotechnology, METU Technocity, Ankara) with the BigDye® Terminator v3.1 Cycle Sequencing Kit, using the ABI 3130xL Genetic Analyzer (Applied Biosystems). Databases were screened for similarities by using BLAST.

Nucleotide sequence accession numbers The nucleotide sequences of the 16S rRNA genes from isolates MF556, MF553, MF548, MF535, MF531, MF495, MF494, MF493, MF489, MF460, MF458, MF404, MF400, MF398, MF380, MF378, MF377, MF376, MF357, MF356, MF354, MF352, MF343, MF322, MF314, MF310, MF308, MF305, MF303, MF293, MF291, MF286, MF282, MF279, MF278, MF276, MF275, MF272, MF271, MF270, MF269, MF268, MF266, MF265, MF253, MF252, MF251, MF250, MF249, MF245, MF244, MF243, MF242, MF241, MF240, MF239, MF237, MF236, MF235, MF233, MF232, MF231, MF230, MF229, MF228, MF226, MF225, MF224, MF223, MF221, MF219, MF215, MF213, MF212, MF210, MF205, MF202, MF201, MF198, MF196, MF195, MF194, MF192, MF187, MF185, MF183, MF180, MF179, MF178, MF177, MF176, MF175, MF172, MF171, MF169, MF167, MF163, MF161, MF159, MF158, MF156, MF152, MF150, MF148, MF147, MF146, MF143, MF141, MF138, MF136, MF128, MF126, MF124, MF118, MF117, MF115, MF114, MF112, MF111, MF107, MF105, MF104, MF103, MF102, MF99, MF92, MF90, MF87, MF86, MF83, MF82, MF78, MF68, MF67, MF63, MF60, MF57, MF56, MF55, MF50, MF48, MF34, MF33, MF31, MF28, MF12, MF11, MF4, MF1, MF14, MF69, and MF214 have been submitted to GenBank and given consecutive accession numbers KJ994358 to KJ994509.

Results and discussion

Chemical and microbiological characteristics of pickle samples

The pH of pickle brine samples ranged from 3.08 to 4.74 with an average of 3.43, and the titratable acidity of pickle brine samples ranged from 0.04 to 1.31 g/100 mL with an average of 0.48 g/100 mL (data not shown). No significant correlation was found between pH and titratable acidity ($r=-0.5463$). This may be due to the different buffering capacities of the brine samples from different vegetable origins. The salt content of the brine samples varied from 1.34 to 6.94 g/100 mL with an average of 3.86 g/100 mL. The wide variations in the

chemical characteristics of the pickle samples may be attributed to different raw materials used in the preparation of collected pickle samples and sampling at different stages of the fermentation process. Another possible explanation for these results may be the different storage conditions in different sampling sites that might affect the natural microflora. These variations also indicate the unstandardized conditions of the industrial production. Accordingly, variable results for titratable acidity, pH, salt, and microbiological quality in pickle samples was also observed by Çon and Karasu (2009). The LAB counts on MRS Agar and M17 Agar plates ranged from <1.00 to 7.75 and from 2.52 to 7.94 log CFU/mL, respectively (data not shown). A total of 557 isolates growing on MRS and M17 Agar were obtained from pickle samples. One hundred and fifty two Gram-positive and catalase- and oxidase-negative isolates were regarded as presumptive LAB and were further characterized by phenotypic and genotypic methods.

Comparison of the classical biochemical methods and the API system

One hundred and fifty two presumptive LAB isolates that were randomly isolated from the fifty three pickle samples were classified into eight preliminary groups (groups A–H) on the basis of key phenotypic characteristics including cell morphology, CO_2 production from glucose, growth at 10 and 45 °C, growth in 6.5 % NaCl, and growth at pH 9.6 (Table 1). This grouping was performed by using a classification scheme derived from taxonomic data on Axelsson 2004. The arginine hydrolysis test was also used as an identification tool (data not shown).

Group A was composed of rod-shaped and heterofermentative LAB. This group comprised obligately heterofermentative lactobacilli (group III) including *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus fermentum*, and *Lactobacillus reuteri*. Forty eight isolates belonged to group A. Group B contained 48 isolates that were rod shaped and homofermentative. This group comprised obligately homofermentative (group I) including *Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, *Lactobacillus helveticus*, and *Lactobacillus salivarius* and facultatively heterofermentative (group II) including *Lactobacillus casei*, *Lactobacillus curvatus*, *Lactobacillus plantarum*, and *Lactobacillus sakei*. Group C was composed of heterofermentative cocci that were able to grow at 10 °C, but not at 45 °C. This group showed variable growth characteristic in 6.5 % NaCl and at pH 9.6. Possible members of this group were the genera *Leuconostoc*, *Oenococcus*, and *Weissella*. None of the tested isolates had exactly the same phenotypic characteristics with group C. Only three isolates were heterofermentative cocci, but had growth characteristics different from those of group C. Group D represented the *Enterococcus* genus. It was composed of homofermentative cocci that were able to grow at both 10 and 45 °C, at pH 9.6, and in 6.5 % NaCl. This group did not include any isolate. Group E comprised 53 isolates that were cocci shaped and homofermentative. Except for one isolate, none of the isolates in this group were able to grow at pH 9.6. Other characteristics in terms of growth at 10 and 45 °C and in 6.5 % NaCl were variable within *Pediococcus* group. Group F, containing no isolates, was ascribed to the genera *Aerococcus* and *Tetragenococcus*. This group was defined as being homofermentative, coccoid LAB growing at 10 °C, at pH 9.6, and in 6.5 % NaCl, but not at 45 °C. Groups G and H did not include any isolate. They shared the common characteristics of being homofermentative cocci and non-growing at pH 9.6, and in 6.5 % NaCl. Group G (*Lactococcus*, *Vagococcus*) could be distinguished from group H (*Streptococcus*, *Pediococcus*) by the ability to grow at 10 °C.

One hundred and fifty two isolates yielded four preliminary groups within the eight defined groups. The preliminary identification results obtained by classical biochemical methods were then compared with those of the API 50 CHL test strips. For the interpretation of API results, a percentage of similarity greater than 90 % was considered as acceptable for identification at species level. Otherwise, the results were taken as doubtful. Thirty two out of the 152 tested isolates yielded an uninterpretable or doubtful API profile.

In accordance with the results of classical phenotypic tests, the isolates belonging to group A were identified as *L. brevis* ($n=34$) and *L. buchneri* ($n=8$) through the API system. Additionally, two isolates were identified as *Lactobacillus pentosus* and one as *L. salivarius* although they produced gas from glucose. These two species are known as homofermentative lactobacilli (Axelsson 2004). Three isolates in this group yielded doubtful API profile.

As shown in Table 1, group B was a heterogeneous group of LAB. There was a consistency between the results of API and classical tests for the identification of 27 isolates in this group including *L. plantarum* ($n=25$) and *L. pentosus* ($n=2$). However, the API identification results of 16 isolates did not match those of classical biochemical tests. Thirteen isolates were identified as *L. brevis* by API although they did not produce gas from glucose. Three isolates that were rod shaped based on microscopic examination were identified as *Pediococcus damnosus* (isolate nos. MF215 and MF115) and *Weissella confusa* (isolate no. MF104) by API. However, some of the physiological characteristics including being obligate homofermentative non-growing at pH 9.6 and inability to hydrolyze arginine of isolate nos. MF215 and MF115 matched those of *P. damnosus* species (data not shown) (Axelsson 2004; Hutkins 2006). It was reported that *Weissella* strains could also be rod shaped. Moreover, isolate no. MF104 possessed a few phenotypic traits of the genus *Weissella* in terms of non-growing at 45 °C and pH 9.6, but it

Table 1 Phenotypic grouping of the LAB strains isolated from pickles

Preliminary group	A	B	C	D	E	F	G	H
Number of isolates	48	48	3[a]	–	53[b]	–	–	–
Presumptive identification	Group III, obligately heterofermentative lactobacilli • L. brevis • L. buchneri • L. fermentum • L. reuteri	Group I, obligately homofermentative or Group II, facultatively heterofermentative lactobacilli • L. acidophilus • L. delbrueckii • L. helveticus • L. salivarius • L. casei • L. curvatus • L. plantarum • L. sakei	Leuconostoc Oenococcus Weissella	Enterococcus	Pediococcus	Aerococcus Tetragenococcus	Lactococcus Vagococcus	Streptococcus Pediococcus
Phenotypic characteristics								
Cell morphology	R	R	C	C	C	C	C	C
CO_2 from glucose	+	–	+	–	–	–	–	–
Growth at 10 °C	NS	NS	+	+	±	+	+	–, ±
Growth at 45 °C	NS	NS	–	+	±	–	–	±
Growth in 6.5 % NaCl	NS	NS	±	+	±	+	–	–, ±
Growth at pH 9.6	NS	NS	–	+	–	+	–	–
API identification	L. brevis (34) L. buchneri (8) L. pentosus (2) L. salivarius (1) Doubtful API profile (3)	L. plantarum (25) L. brevis (13) L. pentosus (2) P. damnosus (2) W. confusa (1) C. maltaromaticum (1) Doubtful API profile (4)	L. brevis (1) L. plantarum (1) L. buchneri (1)	–	L. acidophilus (11) P. damnosus (9) L. plantarum (6) L. brevis (1) Pediococcus spp. (1) Doubtful API profile (25)			

R rod, C Cocci, NS not specified

[a] The characteristics of three isolates in group C; growth at 10 °C (−), growth at 45 °C (two isolates,−)(one isolate, +), growth in 6.5 % NaCl (+), growth at pH 9.6 (−)

[b] One isolate in group E was able to grow at pH 9.6

did not produce gas from glucose and did not grow at 10 °C (data not shown), in contrast to *Weissella* species (Axelsson 2004). An additional isolate was found to belong to *Carnobacterium maltaromaticum*. This isolate possessed some of the key characteristics of the genus *Carnobacterium* (data not shown) (Schillinger and Holzapfel 1995; Axelsson 2004; Hutkins 2006). Four isolates in this group yielded doubtful API profile.

Three isolates that tentatively belonged to group C were identified as *L. brevis*, *L. plantarum*, and *L. buchneri* by the use of API 50 CH. The traditional biochemical analyses did not give reliable identification results for these strains.

The isolates in group E that were presumptively assigned to *Pediococcus* were identified as *L. acidophilus* (*n*=11), *P. damnosus* (*n*=9), *L. plantarum* (*n*=6), *L. brevis* (*n*=1) and *Pediococcus* spp. (*n*=1) due to their carbohydrate fermentation patterns by API 50 CH. Twenty-five isolates in this group gave doubtful API profile with a percentage of similarity below 90 %. Eighteen isolates belonging to group E were identified as *L. acidophilus* (*n*=11), *L. plantarum* (*n*=6), and *L. brevis* (*n*=1) by API 50 CH although they appeared coccoid. This could be explained by morphological variations that could occur within some *Lactobacillus* species (Hammes and Hertel 2006).

Genotypic characterization

In order to corroborate the phenotypic characterization, the 16S rRNA sequencing of 152 isolates was also performed. The two selected primers, namely F27 and R1492, were used to amplify the 16S rRNA fragment of LAB isolates resulting in an amplicon of approximately 1600 bp in size (data not shown). Differences in the species distribution of LAB associated with pickles were defined between the results of API and 16S rRNA sequencing. Following API, the most abundant species was found to be *L. brevis* whereas *P. ethanolidurans* based on 16S rRNA sequencing. Table 2 summarizes the results of the 16S rRNA identification and compares them with the API identification results. The API 50 CHL test coincided with the 16S rRNA results in 71 out of the 152 tested isolates, indicating that API gave unreliable identification results. Consistency between these two methods was observed for *L. brevis* (*n*=32), *L. plantarum* (*n*=30), *L. buchneri* (*n*=8), and *Pediococcus* spp. (*n*=1). In general, the API test failed to identify *Pediococcus* species. Divergent identification results were obtained for the remaining 81 isolates.

As shown in Table 2, 14 isolates identified as *L. brevis* in group A were assigned to *Lactobacillus. namurensis* (6), *L. buchneri* (3), *P. ethanolidurans* (2), *Lactobacillus dioolivorans* (1), *L. plantarum* (1), *Lactobacillus parabrevis* (1) by sequencing of the 16S rRNA gene. This result clearly indicates that the discrimination of these species by

phenotypic methods is very difficult. In group A, isolates classified as *L. pentosus* and *L. salivarius* based on API were reidentified as *L. brevis* and *L. buchneri* by the 16S rRNA sequencing which was consistent with preliminary phenotypic identification. With regard to group B results, it seems that the API test failed to distinguish closely related species including *L. brevis* and *L. buchneri* as well as *L. plantarum* and *L. pentosus*. It has been reported that *L. brevis* and *L. buchneri* are phenotypically very similar, except that *L. buchneri* ferments melezitose (Pot et al. 1994). The results of both API and 16S rRNA sequencing coincided exactly in group C isolates. The group E isolates were mostly found to be *Pediococcus* genus (except for six isolates) which was supported by classical biochemical identification. In group E, the API identification of four isolates which were identified as *L. plantarum* was in agreement with 16S rRNA sequencing. It is worthy of note that *Pediococcus* species were incorrectly identified as *L. acidophilus* by the API 50 CHL test. In accordance with this result, Boyd et al. (2005) reported that some species of lactobacilli were erroneously identified as *L. acidophilus*.

In general, the discrepancy between the results of phenotypic and genotypic identification was in evidence. Even in microscopic examination, misleading morphological results were obtained. This could be explained by the extensive morphological heterogeneity within LAB. Remarkably, *Lactobacillus* is the most physiologically, biochemically diverse group among LAB (Hutkins 2006). Some heterofermentative lactobacilli may appear as cocci, which make the discrimination difficult (Pot et al. 1994).

It was revealed that a variety of LAB species were found in pickle samples. On the basis of 16S rRNA sequencing, the species distribution of LAB associated with pickle samples were as follows: *P. ethanolidurans* (46 isolates, 30.3 %), *L. brevis* (37 isolates, 24.3 %), *L. plantarum* (37 isolates, 24.3 %), *L. buchneri* (15 isolates, 9.9 %), *Pediococcus parvulus* (8 isolates, 5.3 %), *L. namurensis* (6 isolates, 3.9 %), *L. dioolivorans* (1 isolate, 0.7 %), *L. parabrevis* (1 isolate, 0.7 %), and *Enterococcus casseliflavus* (1 isolate, 0.7 %). Unexpectedly, *P. ethanolidurans* was found to be the most predominant LAB species in pickles. Yu et al. (2012) reported that they isolated a small amount of *P. ethanolidurans* strains from naturally fermented pickles. Furthermore, salt-tolerant amylolytic LAB strain *P. ethanolidurans* was isolated from Japanese pickles (*nuka-zuke*) by Iuchi et al. (2012). In recent publications, *P. ethanolidurans* and *L. buchneri* were reported to be involved in the onset of secondary fermentations that lead to fermented cucumber spoilage (Franco et al. 2012; Franco and Pérez-Díaz 2012; Johanningsmeier and Mcfeeters 2013). Since the samples in this study were collected at the various stages of fermentation, it could be likely that some of the samples proceeded to secondary fermentation. Moreover,

Table 2 Comparison of API and 16S rRNA identifications of the LAB strains isolated from pickles

Preliminary group based on classical phenotypic tests	API identification	16S rRNA gene sequencing
A	*L. brevis* (34)	*L. brevis* (20)
		L. namurensis (6)
		L. buchneri (3)
		P. ethanolidurans (2)
		L. diolivorans (1)
		L. plantarum (1)
		L. parabrevis (1)
	L. buchneri (8)	*L. buchneri* (7)
		P. ethanolidurans (1)
	L. pentosus (2)	*L. brevis* (2)
	L. salivarius (1)	*L. buchneri* (1)
	Doubtful API profile (3)	*L. brevis* (1)
		L. buchneri (1)
		P. parvulus (1)
B	*L. plantarum* (25)	*L. plantarum* (25)
	L. brevis (13)	*L. brevis* (11)
		L. buchneri (1)
		L. plantarum (1)
	L. pentosus (2)	*L. plantarum* (1)
		L. brevis (1)
	P. damnosus (2)	*P. ethanolidurans* (2)
	W. confusa (1)	*P. ethanolidurans* (1)
	C. maltaromaticum (1)	*L. plantarum* (1)
	Doubtful API profile (4)	*L. plantarum* (3)
		L. brevis (1)
C	*L. brevis* (1)	*L. brevis* (1)
	L. plantarum (1)	*L. plantarum* (1)
	L. buchneri (1)	*L. buchneri* (1)
E	*L. acidophilus* (11)	*P. ethanolidurans* (10)
		P. parvulus (1)
	P. damnosus (9)	*P. ethanolidurans* (8)
		P. parvulus (1)
	L. plantarum (6)	*P. ethanolidurans* (2)
		L. plantarum (4)
	L. brevis (1)	*P. ethanolidurans* (1)
	Pediococcus spp. (1)	*P. ethanolidurans* (1)
	Doubtful API profile (25)	*P. ethanolidurans* (18)
		P. parvulus (5)
		L. buchneri (1)
		E. casseliflavus (1)

Pediococcus spp. along with *L. plantarum*, which are more acid-tolerant species, has generally been known to predominate at the end of the primary fermentation (Singh and Ramesh 2008; Franco and Pérez-Díaz 2012) and survive during storage (Franco and Pérez-Díaz 2012). Also, *P. ethanolidurans* isolated by Liu et al. (2006) was reported to be resistance to harsh conditions including high ethanol and low sugar concentrations. Although *Leuconostoc mesenteroides* is also considered to be the one of main LAB species present in vegetable fermentations, it could not be
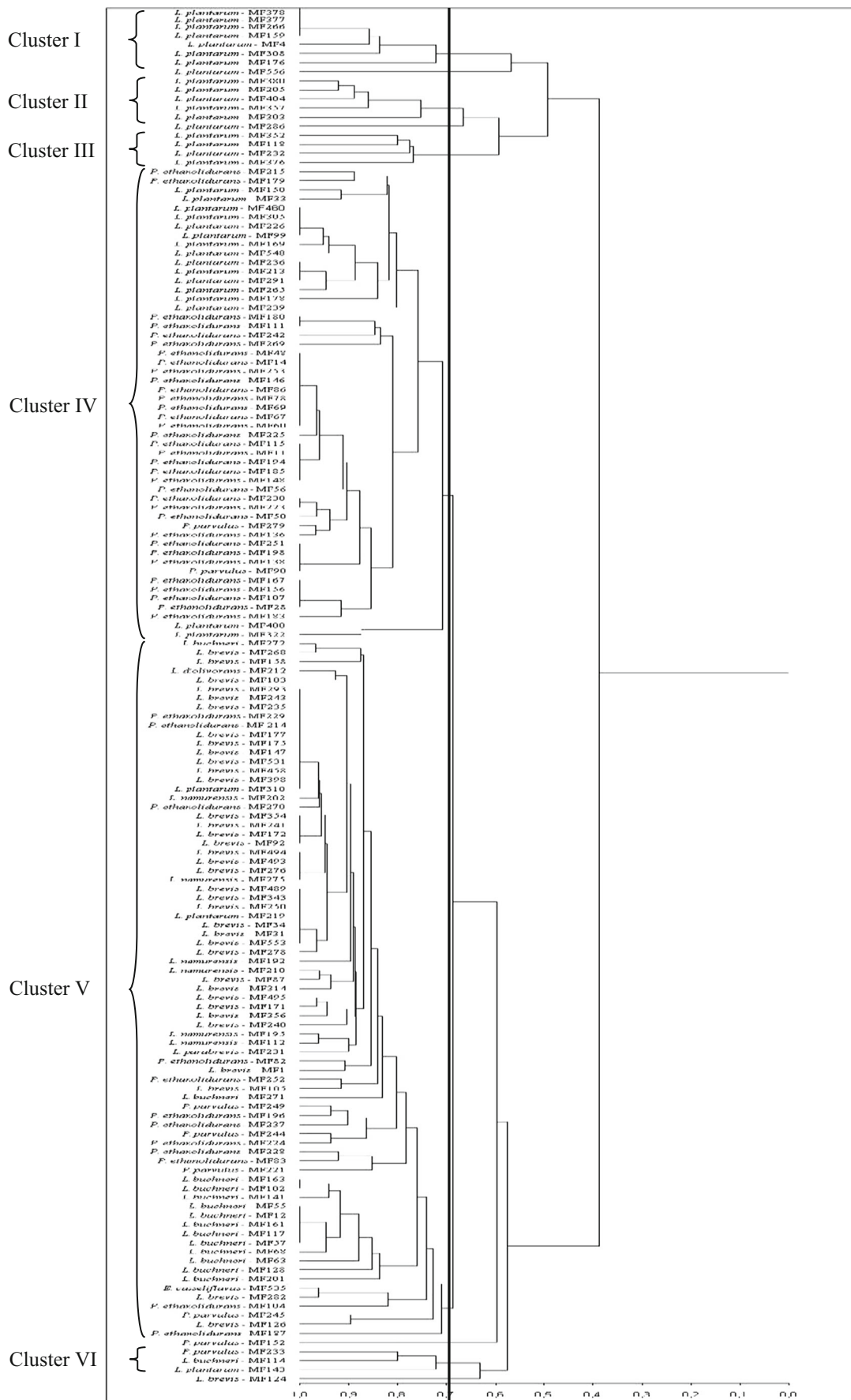
detected in any of the pickle samples either by the API test or the 16S rRNA sequence analysis. A possible explanation for this might be that *Leuconostocs* thrive during the initial stages of fermentation (Singh and Ramesh 2008; Kabak and Dobson 2011). As we collected the samples randomly without following a time-dependent basis to reflect the dynamic changes of LAB microflora during fermentation, it could be possible to miss some of the species involved in the early stage of fermentation. Following *P. ethanolidurans*, *L. brevis* and *L. plantarum* constituted the majority of the LAB species in our study. These two species have been reported to be the main LAB involved in vegetable fermentations including pickles, sauerkraut, olives, and kimchi (Hutkins 2006). Tamang et al. (2005) reported that the major strains of LAB isolated from traditionally fermented vegetable products gundruk, sinki, khalpi, and inziangsang collected from the Northeast India were identified as *L. brevis*, *L. plantarum*, *Pediococcus pentosaceus*, *Pediococcus acidilactici*, and *Leuconostoc fallax*. *L. plantarum* has frequently been reported to be the predominant species in natural vegetable fermentations (Sánchez et al. 2000; Çetin 2011; Yu et al. 2012; Xiong et al. 2012) and could be isolated not only in the process of fermentation but also in the storage period (Tamminen et al. 2004). The predominance of *L. plantarum* in vegetable fermentations has generally been attributed to its distinct features including high resistance to acid and high production of lactic acid (Sánchez et al. 2000; Çon and Karasu 2009; Kabak and Dobson 2011; Xiong et al. 2012). In some previous studies, the presence of *E. casseliflavus* has been reported in raw cucumbers (Chen et al. 2012) as well as fermented cucumbers (Reina et al. 2005). *P. parvulus* has been associated with sauerkraut, fermented vegetables, fermented beans, beer, cider, and wine (Simpson and Taguchi 1995). Six isolates belonging to *P. parvulus* were isolated from the pickles of the Çubuk region in our study. As they are known to tolerate ethanol, it might also be possible to use these isolates for developing malolactic cultures in future investigations. *L. namurensis* was originally isolated from a traditional Belgian sourdough and belongs to the *L. buchneri* group, with *Lactobacillus zymae*, *Lactobacillus acidifarinae*, and *Lactobacillus spicheri* being the closest relatives (Scheirlinck et al. 2007). This species was also isolated from nukadoko, which is a fermented rice bran bed traditionally used in Japan for pickling vegetables (Sakamoto et al. 2011; Kato et al. 2014). *L. namurensis* was found to produce significant amounts of lactic acid, suggesting that it may contribute to the preservation of fermented products (Kato et al. 2014). *L. diolivorans* isolated from maize silage was proposed by Krooneman et al. (2002). *L. diolivorans* which belongs to

L. buchneri group has obligately heterofermentative phenotype (Krooneman et al. 2002). *L. buchneri* has been known to convert lactic acid into 1,2-propanediol (Breidt et al. 2013), and subsequently, 2-propanediol is converted into propionate by *L. diolivorans* (Krooneman et al. 2002). Therefore, Zhang et al. (2010) suggested that propionate production by cocultures of *L. buchneri* and *L. diolivorans* could be applied for bread preservation due to the antifungal capacity of the propionate. From this point of view, the *L. buchneri* and *L. diolivorans* isolates in our study could be used for similar purposes. Vancanneyt et al. (2006) reclassified two *L. brevis* strains which were isolated from cheese and wheat as *L. parabrevis*. Some of the described phenotypic characteristics of *L. parabrevis*, including being heterofermentative, growing at 15 °C and in 6.5 % NaCl, but not at 45 °C, were consistent with those of our isolate (data not shown).

SDS-PAGE profiles

The comparison of whole-cell protein patterns obtained by highly standardized SDS-PAGE has proven to be a reliable method for the discrimination of closely related species (Pot et al. 1994; Vandamme et al. 1996; Hammes and Hertel 2006). Because of the lack of reference LAB strains in our laboratory, SDS-Page profiles of the isolates were used as an adjunct method rather than an identification tool. With this scope, cluster analysis was performed within the tested isolates. The UPGMA dendrogram obtained by whole-cell protein patterns is shown in Fig. 1. All isolates were clustered together at ca. 40 % similarity. When a similarity level of ca. 70 % was used, the isolates (with few exceptions, MF556, MF286, MF152, MF124) classified into six clusters. The isolates produced 1–21 protein bands of molar mass ranging from 7 to 250 kDa. Clusters I, II, and III totally contained the isolates of *L. plantarum* as identified by the 16S rRNA sequencing. In general, *L. plantarum* strains in our study give fewer blurry bands that make the discrimination ambiguous. Thus, *L. plantarum* species fell into all clusters. *P. ethanolidurans* and *P. parvulus* species mainly grouped into cluster IV. Cluster V comprised isolates belonging to *L. brevis*, *L. buchneri*, *L. namurensis*, *L. diolivorans*, *L. parabrevis*, *P. ethanolidurans*, *P. parvulus*, and *E. casseliflavus*. The high similarity level obtained between these species indicated that they cannot be distinguished by SDS-PAGE of whole-cell proteins. In summary, it is hard to find a correlation between the results of whole-cell SDS profiles and 16S rRNA sequencing without the use of reference LAB strains.

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