Determination of Antagonistic Starter Cultures for Pickle and Olive Fermentation Processes

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Abstract

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In the present study, the main purpose was the selection of antagonistic starter cultures for pickle and olive fermentation processes. The chemical and microbiological properties of fermented 70 pickle and 16 olive samples collected from the province of the west part of Turkey were analysed. Subsequently, lactic acid bacteria strains producing bacteriocin-like metabolites were isolated and identified. From 86 samples, 16 isolates were chosen, depending on their partial antimicrobial activity against at least one selected indicator. 13 out of this 16 isolates were identified as *Lactobacillus plantarum* whereas 3 of them proved to be *Lactobacillus pentosus*. Moreover, all the relevant isolates were found to be potent acid producers. All these results obviously suggest that the isolated *Lactobacillus plantarum* 9 and 25 were appropriate for them to be proposed as starter cultures in fermented pickle and olive production.

Keywords: pickle; olive; *Lactobacillus*; antimicrobial activity

Fermented pickle belongs to the products stabilised with salt and lactic acid which is accumulated by lactic acid bacteria (LAB) fermentation (Aktan *et al*. 1999). However, the products prepared in diluted acetic acid solution spiced with some flavour or aroma herbals are also included under this definition (Anonymous 1993). Olives is another fermented product being important for human nutrition, due to its high oil content (33%), fibrous compounds, protein, minerals, organic acids, phenolic compounds, pectic compounds, and carotene which are eventually both quantitatively and qualitatively changed during the maturation and growth phases of olives (Aktan & Kalkan 2000).

Some members of the lactic acid bacteria have a considerable importance in the fermentation of pickle and olives. They provide a rapid acid accumulation in the raw material with the production of lactic and several organic acids. Furthermore, they can also produce various aroma components, bacteriocins, and exopolysaccharides. These metabolic products contribute to the development of some characteristic properties such as taste, visual appearance, texture, shelf life, and safety (Holzapfel 1997; Leroy & de Vuyst 2004). The pickles obtained by fermentation, have been accepted to have a protective effect for human health as observed in the case of gut (Şahin & Akbaş 2001). Moreover, they are very important nutritionally as they play a role in the biosynthesis of vitamins, essential amino acids, and proteins (Giraffa 2004).

For selecting microorganisms for starter cultures to be used in fermented foods, it is expected that the strains should have some characteristics, such as: adapting easily to the raw material and process, developing sensory quality, extending shelf-life,

reducing the processing time and energy during the production, inhibiting food related pathogenic microorganisms as well as having probiotic, non-pathogenic, and non-toxigenic properties. According to these criteria, important LAB finding applications in fermented foods are given in Table 1. From these species, *Lactobacillus plantarum* is dominant during the whole fermentation process since it is the most acid resistant microorganism of these species (Daeschel *et al*. 1988).

Besides providing standard and quality food production, the use of starter cultures with the stated properties also gives rise to the degradation of antinutritional factors, improvement of protein digestibility and bio-availability of micronutrients, and nutritional enrichment of food through the biosynthesis of vitamins, essential amino acids, and proteins. Moreover, some members of lactic acid bacteria carry out detoxification of toxic compounds and degradation of mycotoxins in specific cases (Holzapfel 1997, 2002) and therefore can reduce the health risks. They also enhance the shelf life of foods by inhibiting the flora responsible for the undesirable taste by spoiling the food (Ross *et al.* 2002).

Leroy and de Vuyst (2004) found that, LAB which have been placed on the market for fermented food production in recent years, increase food safety by producing organic acids and antimicrobial substances, providing probiotic properties, producing sugar polymers, sweetening aromatic compounds, vitamins, or useful enzymes. Tolonen *et al*. (2004) also demonstrated that it is possible to produce a standard and quality product in a short time by the use of starter culture in fermented food production. The microorganisms used in the production of pickle and olive have been identified as *L. plantarum, Leu. mesenteroides* ssp. *mesenteroides* and *Pediococcus cerevisiae.*

The specific antagonistic activity of LAB in various foods existing naturally or added as starter cultures to spoiling microorganisms or to foodborne pathogens is due to their production of organic acids, hydrogen peroxide, carbon dioxide, diacetyl, ethanol, bacteriocins, and similar compounds (Caplice & Fitzgerald 1999). The LAB, although consisting of a number of diverse genera, are grouped as either homofermenters or heterofermenters, based on the final product of the glucose fermentation. The homofermenters produce lactic acid as the major product of glucose fermentation. The heterofermenters produce, besides lactic acid, a number of products, including carbon dioxide, acetic acid, and ethanol from coming the fermentation of glucose (Carr *et al*. 2002; Leroy & de Vuyst 2004). The direct antimicrobial effects of lactic, acetic, and propionic acids are well known. The antagonism is believed to result from the action of the acids on the bacterial cytoplasmic membrane, interfering with the maintenance of the membrane potential and inhibiting the active transport (Caplice & Fitzgerald 1999). Nonetheless, lactic acid is ineffective to mould and yeast at pH 5, but it is a good inhibitor of spore forming bacteria. Acetic acid also demonstrates antimicrobial activity against many bacteria including coliforms and salmonella (Çon & Gökalp 2001).

By taking into account this situation, it was planned to study the antagonistic activity of bacteriocins and other metabolites of LAB isolated from traditionally produced pickles and olives,

Table 1. Lactic acid bacteria in fermented vegetable products (Caplice & Fitzgerald 1999)

Product	Country	Microorganism	Raw material
Sauerkraut	international	*Leuconostoc mesenteroides* *Lactobacillus brevis* *Lactobacillus plantarum* *Lactobacillus curvatus* *Lactobacillus sake*	cabbage
Pickle	international	*Pediococcus cerevisiae* *Lactobacillus plantarum*	cucumber
Olive	mediterranean countries	*Leuconostoc mesenteroides* *Lactobacillus plantarum*	olive

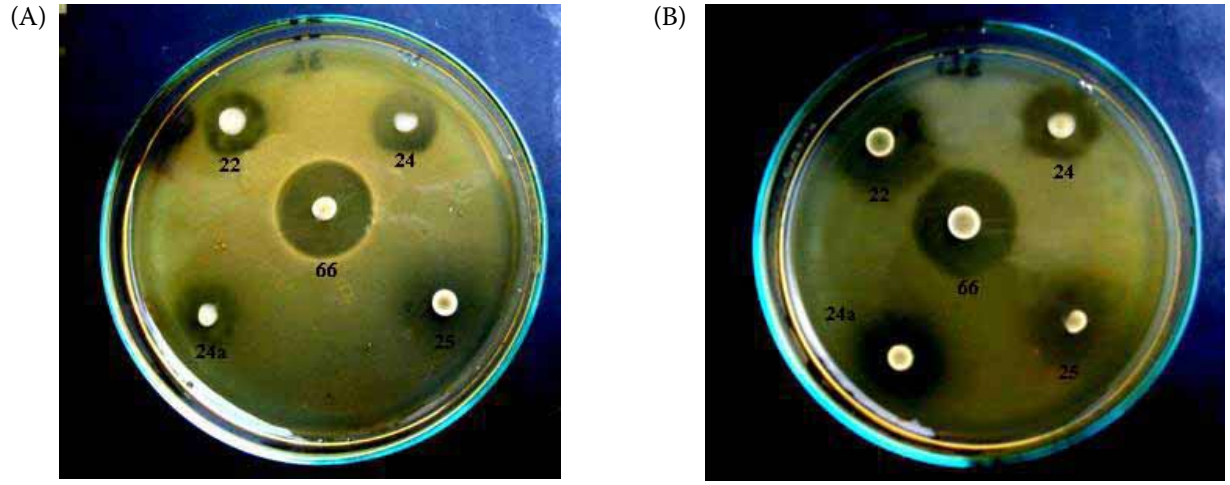

Figure 1. Antimicrobial activity of *Lb. plantarum* 22, 24, 24a, 25 and 66: (A) against *Lb. sake* Lb790; (B) against *L. monocytogenes* Li1

and to determine the ability of microorganisms to produce acid for the product quality. In this way, it would be possible to determine the starter culture possessing antimicrobial activity for standard, quality pickles and olives production.

MATERIAL AND METHODS

Material. In this study, 70 pickles (prepared by using pepper, bean, cabbage, beet, cucumber, grape, and tomato separately or in mixtures) and 16 olive samples were randomly taken from plants and households in the province of Aydın and Denizli.

Bacterial strains and culture media. MRS agar (Merck 1.10661) was used for the isolation and identification of LAB from pickles and olives samples (Sanchez *et al*. 2000; Santos *et al.* 2003). MRS agar containing 0.2% glucose (w/v) (MRS-0.2), MRS-0.2 soft agar containing 0.7% agar (w/v), Tryptone Soya Soft Agar (TSYE) containing Tryptone Soya Broth (Oxoid CM129) + Yeast Extract (Merck 1.03753) + 0.7% agar (w/v) and were used to determine antimicrobial activity of the isolated strains (Şimşek *et al*. 2006). Proteolytic activity of the strains was determined using 10% skim milk (Şimşek 2003). All the purified strains of LAB were stored in skim milk (Oxoid L31) containing 15% (v/v) glycerol at –20°C (Sanchez *et al*. 2000; Yüksekdağ *et al*. 2004b), submerging cultures at +4°C on MRS agar, and after applying lyophilisation (Lewus *et al*. 1991; Yüksekdağ *et al*. 2004).

Lactobacillus sake Lb706 was used as a bacteriocin producing strain and *Lb. sake* Lb706-A was used as a bacteriocin non-producing strain. *Listeria monocytogenes* Li6, *L. monocytogenes* Li1, *L. sake* Lb790, *E. coli, P. vulgaris, Y. lipolitica, A. hydrophila* and *E. faecium* were used as sensitive strains (Table 2).

Microbiological and chemical analyses of fermented pickle and olive samples. Fermented pickle and olive samples provided under aseptic conditions from plants and houses were opened in laboratory and samples were taken for microbiological and chemical analyses carried out in parallels. The samples were taken after homogenisation was carried out by a thorough shaking of the jugs.

LAB, total aerobic mesophilic bacteria (TAMB), mould-yeast (MY), and coliform counts were determined according to (Tassou *et al*. 2002; Şimşek *et al*. 2006). pH values of the samples were determined by a method described by Tassou *et al*. (2002) snd Şimşek (2003), titretable acid amounts (as % acetic acid) and salt contents were determined by the methods of Aktan and Kalkan (2000).

Isolation, identification, antimicrobial activity and acid producing ability of lactic acid bacteria. The Isolation from and identification of LAB in the samples were carried out and the antimicrobial activity spectrum of the strains and the total titretable acid amounts (as % lactic acid) produced by the strains were determined according to the methods given by (Çon 1995).

For partial identification, LAB were grown in 10 ml MRS broth at 30°C for 24 h and examined for the gas production from glucose (Randazzo *et al*. 2004), further arginine hydrolysis test (Çon

Table 2. Reference and indicator bacteria strains used in the research

Strain	Aim	Source
Lactobacillus sake Lb706	reference	The Institute of Federal Meat Research, Kulmbach
Lactobacillus sake Lb706-A	reference	The Institute of Federal Meat Research, Kulmbach
Lactobacillus sake Lb790	indicator	The Institute of Federal Meat Research, Kulmbach
Listeria monocytogenes Li6	indicator	The Institute of Federal Meat Research, Kulmbach
Enterococcus faecium NRRL B-2355	indicator	Aegean Univiversity, Agriculture Faculty, Milk Technology Department
Escherichia coli ATCC39403	indicator	Pamukkale University, Medical Faculty, Microbiology Research Laboratory
P. vulgaris RSSK 96025	indicator	Pamukkale University, Food Engineering Department
Yersinia lipolitica NCAIM Y.00591	indicator	Aegean University, Agriculture Faculty, Milk Technology Department.
A. hydrophila NRRL B-426	indicator	Aegean University, Agriculture Faculty, Milk Technology Department

1995), catalase test (Kim *et al*. 2001; Carr *et al*. 2002) and the growth in MRS broth at 15°C and 45°C were followed for seven days. Carbohydrate fermentation profiles of each strain was determined by using API 50 CH strips and API 50 CHL medium (Bio Merieux) for the ultimate identification, the fermentation results being recorded and evaluated after 24 h, 48 h and 72 hours.

RESULTS

Chemical and microbiological properties of pickles and olives

It was found that the fermented samples (seventy pickles and sixteen olive samples), analysed in this study, had significantly different microbiological and chemical characteristics. The chemical results of the pickle samples showed that pH ranged between 3.50–3.94 with the average of 3.53, titretable acid amount ranged from 0.18% to 4.41% acetic acid having an average of 1.60% acetic acid, salt concentration ranged from 0.39% to 9.89% with an average of 3.96%. These results were within the ranges obtained by (Johanningsmeier *et al*. 2004; Tolonen *et al*. 2004) and the results and standards given by the Turkish Standards Institute (TSI). With the olive samples, pH ranged from 2.81 to 4.84 with an average of 3.77, titretable acid amount were 0.20–2.12% acetic acid with an average of 0.91% acetic acid, and salt concentration was 0.18–10.82% with an average of 4.63%. pH values of the olive samples were found to be lower than those reported by (Montano *et al*. 2003; Marsilio *et al*. 2005). The samples with low acid values were expected to have high salt concentrations. In addition to this, with respect to the acid values, salt concentration, and pH values, the olive samples were found appropriate in view of the standards of TSI.

The microbiological counts of LAB in the pickle samples ranged from < 3.00 to 7.80 log CFU/g, the average value being 5.79 log CFU/g, TAMB ranged from < 3.00 to 8.28 log CFU/g with an average of 5.48 log CFU/g, and MY ranged from < 3.00 to 7.85 log CFU/g with an average of 5.24 log CFU/g. The coliform group bacteria were detected in seven samples (10%) with the average counts of 1.83 log CFU/g. These results were within the range obtained by Johanningsmeier *et al*. (2004) but lower then those by Tolonen *et al*. (2004). On the other hand, LAB count of the fermented olive samples ranged between < 3.00-7.15 log CFU/g with an average of 6.20 log CFU/g, TAMB count ranged between 3.95–7.18 log CFU/g with an average of 6.01 log CFU/g, and MY ranged between < 3.00–6.91 log CFU/g with an average of 5.60 log CFU/g. The coliform group bacteria were detected in three (19%) samples having an average count of 1.52 log CFU/g. However, *E. coli* was not detected in any of the samples. The average LAB count results were found to be higher than those reported by Panagou and Katsaboxakis (2006), but they resembled those of (Leal-Sanchez *et al*. 2003;

Table 3. The basic features of isolates and isolation sources

Isolates	Gram painting	Catalase	Arginine hydrolysis	Gas production	Morphology	Isolation Source
Lb. plantarum 2	+	–	–	–	bacillus	red pepper pickle
Lb. plantarum 3	+	–	–	–	bacillus	bean pickle
Lb. pentosus 5	+	–	–	–	bacillus	mixed pickle
Lb. pentosus 6	+	–	–	–	bacillus	green olive
Lb. plantarum 9	+	–	–	–	bacillus	cabbage pickle
Lb. plantarum 11	+	–	–	–	bacillus	cabbage pickle
Lb. plantarum 12	+	–	–	–	bacillus	cabbage pickle
Lb. pentosus 13	+	–	–	–	bacillus	beet arm pickle
Lb. plantarum 18	+	–	–	–	bacillus	cucumber pickle
Lb. plantarum 19	+	–	–	–	bacillus	grape pickle
Lb. plantarum 21	+	–	–	–	bacillus	cabbage pickle
Lb. plantarum 22	+	–	–	–	bacillus	mixed pickle
Lb. plantarum 24	+	–	–	–	bacillus	mixed pickle
Lb. plantarum 24a	+	–	–	–	bacillus	mixed pickle
Lb. plantarum 25	+	–	–	–	bacillus	pepper pickle
Lb. plantarum 66	+	–	–	–	bacillus	tomato pickle

Marsillio *et al.* 2005). YM counts varied within a greater range than those given by Panagou and Katsaboxakis (2006) and the average value was also higher. Coliform bacteria values were similar to those given by (Marsillio *et al.* 2005).

Identification of the isolates

Appropriate dilutions of the fermented pickle and olive samples were plated on MRS agar (Oxoid CM361), and the plates were incubated anaerobically (90% N_2, 10% CO_2) at 30°C for 48–72 hours. The colonies showing antimicrobial zones after pouring the MRS soft agar containing 1% of the indicator strain (*Lb. sake* Lb790) on the MRS plates were isolated as having antimicrobial activity. More than 4000 colonies could be evaluated using this strategy. From these colonies, the isolates which were Gram (+), catalase (–), coccus or bacillus shaped, or non-spore forming were selected and stored as potential LAB with antimicrobial activity for further studies (Çon 1995). After the sampling, 16 isolates having the highest antimicrobial activity were selected using the agar spot test. The basic features of the isolates and isolation sources are shown in Table 3. Carbohydrate fermentation profiles of the isolates showed that 13 out of 16 isolates were homologous to *Lb. plantarum* while 3 of them were homologous to *Lb. pentosus* over 90%, when analysed with the software given by Bio Merieux.

Antimicrobial activity of isolates

The antimicrobial spectra of the lactobacillus strains in their growth lawn are shown in Table 4. All the isolated *Lb. plantarum* strains exhibited medium or high antimicrobial activities against the indicator strains *Lactobacillus sake* Lb790, *Listeria monocytogenes* Li1, and *Listeria monocytogenes* Li6. However, differences were found in antimicrobial activity against *Enterococcus faecium* among the *Lb. plantarum* strains. Out of those *Lb. plantarum* 66 showed the highest antimicrobial activity against all gram positive indicator strains. Among the gram negative indicator strains (*Echerichia coli, Proteus vulgaris, Yersinia lipolitica* and *Aeromanas hydrophila*), *E. coli* was inhibited by *L. plantarum* strains at a higher level than the others while no inhibitory effect could be detected on *Y. lipolitica*. Additionally, a low inhibitory effect was detected on

Table 4. Antimicrobial activity spectra of isolates

Isolates	*Lb. sake* Lb790	*L. monocytogenes*		*E. feacium*	*E. coli*	*Y. lipolitica*	*P. vulgaris*	*A. hydrophila*
		Li1	Li6					
L. plantarum 2	+++	++	++	+	++	–	+	+
L. plantarum 3	+++	++	++	–	+	–	+	–
L. pentosus 5	+++	++	++	++	+	–	+	–
L. pentosus 6	++	++	++	+	–	–	+	–
L. plantarum 9	++	++	++	+	++	–	+	+
L. plantarum 11	++	++	++	+	++	–	+	–
L. plantarum 12	+++	++	++	+	+	–	++	–
L. pentosus 13	++	++	++	+	++	–	+	–
L. plantarum 18	++	++	++	+	++	–	+	–
L. plantarum 19	++	++	++	++	++	–	+	+
L. plantarum 21	++	++	++	++	++	–	+	+
L. plantarum 22	+++	++	++	++	++	–	++	+
L. plantarum 24	++	+	+	–	++	–	+	–
L. plantarum 24a	+++	++	++	+	++	–	+	+
L. plantarum 25	+++	++	++	++	+	–	+	+
L. plantarum 66	+++	+++	++	+++	–	–	+	++

*The thickness of the inhibition zone: – < 0.5 mm; + 0.5–1.0 mm (low effect); ++1.1–3.0 mm (medium effect); +++ > 3.0 mm (high effect)

both *P. vulgaris* and *A. hydrophila* indicator strains. On the other the hand, three *Lb. pentosus* strains isolated showed different levels of the inhibitory effect against all indicator strains except *Y. lipolitica* and *A. hydrophila* which were not inhibited (Table 4). Those *Lb. pentosus* strains exhibited medium antimicrobial effects against *Lb. sake* Lb790, *L. monocytogenes* Li1, and *L. monocytogenes* Li6, and low antimicrobial effects against *E. faecium*, *E. coli* and *P. vulgaris*. These inhibitory activity results obtained with all lactobacillus strains were found similar to those observed with the strains isolated those found with of *Lb. plantarum* strains isolated from sucuk by (Çon & Gökalp 2000). In addition, Santos *et al*. (2003) identified *Lb. plantarum* isolates from olive samples which exhibited inhibitory activity against *L. monocytogenes*.

Total titretable acid amount produced by isolates

The total titretable acid amount produced by the lactobacillus strains isolated from fermented vegetables was followed during 7 days. At the end of the first day, *Lb. plantarum* 9 and 25 and *Lb. pentosus* 13 had produced the highest levels of total titretable acid amount (1.95% lactic acid), followed by the strains *Lb. plantarum* 2 and 3 with the levels of 1.90% lactic acid. Additionally, it was found that all strains were able to produce more than 80% of their total titretable acid amounts after the first day of incubation. Also, the titretable acid amounts produced by *Lb. plantarum* and *Lb. pentosus* strains at the end of the 7th day were found varying very closely from 2.00% to 2.15% lactic acid with an average of 2.06% lactic acid (Table 5).

DISCUSSION

In Turkey, many fermented vegetable products have been produced traditionally by spontaneous microflora which results in unstable and non-standard products. Therefore, starter culture studies could be useful to minimise the fermentation risks and to reach a standard production schedule. The main purpose of this study was

Table 5. Total acid production of isolates in the course ofr seven days

Isolates	1st day		4th day		7th day	
	pH	% acid*	pH	% acid	pH	% acid
L. plantarum 2	3.25	1.90	3.93	1.95	3.85	2.00
L. plantarum 3	3.26	1.85	3.88	1.85	3.96	2.15
L. pentosus 5	ND	ND	ND	ND	ND	ND
L. pentosus 6	3.42	1.60	3.94	1.80	3.95	2.05
L. plantarum 9	3.28	1.95	3.91	1.95	3.93	2.15
L .plantarum 11	3.32	1.80	3.92	1.80	3.92	2.10
L. plantarum 12	3.26	1.80	3.90	1.75	3.94	2.05
L. pentosus 13	3.21	1.95	3.89	1.80	3.91	2.15
L. plantarum 18	3.25	1.60	3.93	1.80	3.95	2.05
L. plantarum 19	3.28	1.75	3.92	1.75	3.98	2.05
L. plantarum 21	3.28	1.80	3.93	1.75	3.95	2.00
L. plantarum 22	3.30	1.75	3.94	1.80	3.95	2.00
L. plantarum 24	3.74	1.75	3.95	1.45	3.97	2.00
L. plantarum 24a	3.27	1.80	3.94	1.75	3.97	2.05
L. plantarum 25	3.69	1.95	3.92	1.70	3.94	2.05
L. plantarum 66	3.32	1.70	3.96	1.70	3.97	2.10
Average	3.34	1.80	3.92	1.78	3.94	2.06

*Total titretable acid amount calculated as % lactic acid; ND – not detected

to isolate and identify potential starter cultures for the production of fermented pickle and olive products. Consequently, traditionally produced different fermented pickle and olive samples were collected from the west part of Turkey as a source of potential lactobacillus strains.

The results indicated that the pickle and olive samples revaled significantly variable values for the titretable acid content in %, pH, salt, and microbiological quality. This could be attributed to the local traditional production habits. Another possibility for this variation is, the storage conditions which might affect particularly the *in vivo* microbial growth. These results are evidence for insufficiency in the standardisation of the industrial production; however, collected fermented vegetable samples are an incomparable source for the isolation and identification of the desirable lactobacillus strains because of their production being carried out with their own spontaneous microflora.

More than 4000 isolates were evaluated throughout the study out of which 32 isolates were selected due to their antimicrobial activity. However, the ultimate selection was done employing agar spot antimicrobial activity tests against several indicators while only 16 of the isolates exhibited higher inhibitory activity. Of the 16 isolates, 13 (81.3%) were affiliated to *Lb. plantarum* and 3 (18.7%) to *Lb. pentosus* with over 90% homology at the API carbohydrate fermentation tests. These results indicated that the basic lactic microflora of fermented vegetables originated from Turkey is composed with mainly of *Lb. plantarum* species similar to those previously reported (Sanchez *et al*. 2000; Tassou *et al*. 2002; Leal-Sanchez *et al*. 2003).

The isolated and identified lactobacillus strains exhibited different levels of antimicrobial activity against the selected indicator strains. Among the isolates, *Lb. plantarum* 2, 12, 22, 24a, 25, 66, and *Lb. pentosus* 5 had higher inhibitory activities compared to the others, suggesting that these strains could be used as starter cultures. Especially, high inhibitory effects of these strains on the pathogen bacteria such as *L. monocytogenes* Li1, Li6, and

E. coli, increase their importance for the industrial applications. Notably, in most studies *Lb. plantarum* strains have been suggested as antagonistic starter cultures for their high lactic acid production and various inhibitory metabolites (Caplice & Fitzgerald 1999; Santos *et al*. 2003).

The ability of a rapid and high acid production has been demanded for lactic cultures to be used as starters in the vegetable fermentation technology (Buckenhüskes 1993). Therefore, *Lb. plantarum* 25 and *Lb. pentosus* 13 may be preferred as starter cultures because they produce most of the total amount of acid in the first day. On the other hand, the acid production levels of the isolated *Lb. plantarum* strains were found to be higher than those reported with *Lb. plantarum* strains originated from sausage, kefir, and sourdough (Yaman *et al*. 1998; Yüksekdağ *et al*. 2004a; Şimşek *et al*. 2006), indicating that the acid production ability of the strains can change depending on the isolation source.

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