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ORIGINAL ARTICLE

Technological and probiotic characteristics of *Lactobacillus plantarum* strains isolated from traditionally produced fermented vegetables

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Abstract The technological and probiotic characteristics of a number of *Lactobacillus plantarum* strains (previously isolated and identified from traditionally produced fermented vegetables) were compared, with the aim of identifying potential starter cultures to be used for the fermentation of vegetables. The *L. plantarum* strains were differentiated by their plasmid profiles; 12 separate strains with different plasmid profiles were examined. Other than survival in different concentrations of NaCl, the technological characteristics of all the strains examined were similar. However, strain-dependent variations in probiotic features were observed, with low pH in particular found to be a restrictive condition for all strains. The results indicated that, among the tested strains, *L. plantarum* 3 and 22 had good technological properties whereas *L. plantarum* 66 was significantly efficient in terms of probiotic features. Overall, *L. plantarum* 22 was found to be the most appropriate strain due to its combination of technological and probiotic properties.

Keywords *Lactobacillus plantarum* · Fermented vegetables · Probiotic · Technological characteristics

Introduction

Preservation of vegetables by fermentation of lactic acid bacteria (LAB) is an ancient practice (Holzapfel 2002). Traditionally, fermented vegetables have been produced by taking advantage of the natural microbiota associated with plant material. However, spontaneous fermentation is uncertain and the quality of the final products varies depending on the fermented material and inherent microbiota. Starter LAB cultures with desirable properties, for instance possessing proteolytic activity, good survival in high saline concentration and after freeze-drying, as well as producing high amounts of lactic acid, aromatic compounds, bacteriocins and exopolysacharrides (EPS), are particularly important for achieving high quality fermented vegetable products and preventing economic losses (Gardner et al. 2001).

Indigenous lactic acid producing microbiota change spontaneously during natural vegetable fermentation and *Lactobacillus* species become abundant towards the end of the fermentation (Caplice and Fitzgerald 1999; Randazzo et al. 2004; Panagou and Katsaboxakis 2006). Within this genus, *Lactobacillus plantarum* is one of the most isolated and identified species due to its ability to tolerate the high acidity and saline content of fermented vegetables, mainly cucumber, olive and sauerkraut. Hence, strains of *L. plantarum* have been considered as convenient starter cultures for use in fermented vegetable production (Sanchez et al. 2000; Panagou and Katsaboxakis 2006). On the other hand, *L. plantarum* strains have also been characterized as probiotic cultures, as they have sufficient resistance towards extreme intestinal conditions (gastric acidity and bile toxicity). Furthermore, it has been shown that some strains possess other probiotic properties, including high β-galactosidase activity and hydrophobicity, that are related to the adhesion ability of these strains to their host (Ouwehand et al. 1999; De Vries et al. 2006; Maragkoudakis et al. 2006).

Dairy products such as fermented milk and yoghurt are often used as carriers for probiotic cultures (Fonden et al.

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2000; Dethlefsen et al. 2006). However, lactose intolerance and the cholesterol content are two drawbacks related to their consumption. In recent years, there has been increasing interest in the use of non-dairy-based probiotic foods for administrating probiotics (Yoon et al. 2006). Recently, attention has focused on the use of fermented vegetables as a food carrier. However, some well-known probiotic cultures (*Lactobacillus rhamnosus* GG, *Bifidobacterium bifidum* ATCC 15696 and *Bifidobacterium longum* ATCC 15708) tested for fermented vegetable production exhibited low cell viability (less than 10^7 CFU/mL) at the time of consumption (Lavermicocca et al. 2005; Yoon et al. 2006). It has been established that survival of probiotic strains in fermented foods is restricted mainly by the presence of salt as well as other factors including oxygen and temperature (Champagne et al. 2005). Therefore, obtaining starter cultures from among the *Lactobacillus* strains that often exist naturally in fermented vegetables, and testing these for potential probiotic characteristics, may make it possible to overcome problems of survival.

In Turkey, many fermented vegetable products are produced by traditional methods relying on fermentation by natural microbiota. In our recent study (Çon and Karasu 2009), we isolated and identified mainly *L. plantarum* strains as the predominant microbiota of different traditionally produced fermented vegetable samples obtained from several Turkish provinces. Therefore, in this study, isolated *L. plantarum* strains were differentiated on their plasmid profiles, and their probiotic and technological characteristics were subsequently compared with the aim of attaining appropriate starter culture strains for delivering probiotics to humans.

Materials and methods

Bacterial strains and culture media

Lactobacillus plantarum strains isolated from traditionally produced fermented vegetables (Çon and Karasu 2009) were obtained from the strain collection of the Department of Food Engineering, University of Pamukkale. The code number and isolation source of each strain is given in Table 1. The strains were cultivated in de Man, Rogosa and Sharpe broth and agar (Merck, Darmstadt, Germany) at 30°C for 48 h. All *L. plantarum* strains were preserved in skim milk (Oxoid L31, Oxoid, Basingstoke, UK) containing 15% (v/v) glycerol at −20°C (Sanchez et al. 2000).

Plasmid analysis of *Lactobacillus plantarum* strains

The plasmid content of isolated *L. plantarum* strains was determined according to the protocol proposed by Anderson and McKay (1983). Plasmid DNA samples were subjected to electrophoresis in 0.7% agarose gels.

Determination of technological properties of *Lactobacillus plantarum* strains

The viabilities of *L. plantarum* strains under different NaCl concentrations (3, 5, 6.5, 8 and 9%) were determined in MRS broth according to the method of Sanchez et al. (2000). Proteolytic activity of strains was determined by measuring the absorbance in 10% skim milk at 650 nm with a spectrophotometer (Shimadzu 1601; http://www.

Table 1 Technological features of *Lactobacillus plantarum* strains

L. plantarum strain	Survival in different NaCl concentrations		Proteolytic activity (mg/mL)	Survival rates after freezing (%)	Survival rates after lyophilisation (%)	Source
	8%	9%				
2	+[a]	−	0.083	91	100	Red pepper
3	+	+	0.063	100	100	Bean
9	+	−	0.075	87	98	Cabbage
11	+	−	0.060	85	93	Cabbage
12	+	−	0.056	81	87	Cabbage
18	+	−	0.066	100	100	Cucumber
19	+	−	0.056	82	84	Grape
21	+	−	0.058	89	87	Mixed
22	+	+	0.073	92	94	Mixed
24	−	−	0.068	100	95	Mixed
25	+	−	0.066	93	82	Pepper
66	+	−	0.062	79	72	Tomato
Average			0.065	82.5	91.1	

Data are the means of duplicate trials

[a] + Growth, − no growth

shimadzu.com/) following incubation at 30°C for 42 h (Citi et al. 1963). The results were expressed as milligrams/milliliter tyrosine by means of reference to a calibration curve. The method proposed by G-Allegria et al. (2004) to determine the viability levels of *L. plantarum* strains against freezing and lyophilisation was followed with minor modifications. Cells were centrifuged after incubation in MRS medium at 30°C. Pellets were then resuspended in 0.5 ml sterilized skim milk and frozen at −70°C followed by lyophilisation (Thermo Savant Modulyo D; http://www.thermo.com/). The viability rates of *Lactobacillus* strains after freezing and lyophilisation were calculated as a percentage of the initial count of each strain.

Survival under conditions simulating the human gastro-intestinal tract

Resistance of the *L. plantarum* strains to gastric transit was determined in a simulated gastric juice prepared by suspending pepsin (0.3% w/v) in sterile saline (0.5% w/v) and adjusting the pH to 2.0 and 3.0 separately with concentrated HCl as described previously (Gardiner et al. 1999). Resistance was assessed in terms of viable colony counts, enumerated after incubation at 37°C for 0 and 3 h.

Tolerance to bile salts was tested at 37°C by inoculation of fresh cultures into MRS broth enriched with 1 and 4% ox-bile (w/v; Oxoid). Absorbance was measured after 10 and 20 h of incubation. The rate (%) of resistance was calculated by comparing the absorbance of cell growth in MRS with and without ox-bile (Vinderola and Reinheimer 2003).

β-Galactosidase activity and antibiotic resistance

The substrate O-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma, St. Louis, MO) was used to determine β-galactosidase activity. The yellow color formed after incubating for 3–4 h at 37°C represented β-galactosidase activity.

To determine the antibiotic resistance of *L. plantarum* strains, a method proposed by Charteris et al. (1998) was followed with some modifications. Antibiotic discs were placed and the diameter of inhibition (mm) was measured after a 24-h incubation. Characterization of sensitivity/resistance was performed and evaluated according to the guidelines and breakpoints of the European Commission (SCAN, Directorate C, Scientific Opinions, 2002) and the European Food Safety Authority (EFSA 2005).

Hydrophobicity assay

Fresh cultures of *L. plantarum* strains were centrifuged at 8,000 *g* for 10 min, and the absorbance was adjusted to 1 at 560 nm after washing the cells three times with PBS. Subsequently, 0.6 ml α-hexadecane (Merck) was added to 3 ml cell suspension. Tubes were thoroughly mixed on a vortex for 2 min and the hydrocarbon phase was allowed to separate completely (approximately 1 h at 37°C). Finally, the aqueous phase was removed carefully and transferred to a 1 ml cuvette for measuring the absorbance at 560 nm. The percentage of hydrophobicity was calculated with the following formula:

$$H\% = [(A_0 - A)/A_0] \times 100$$

where A_0 and A are the absorbance values of the aqueous phase before and after addition of α-hexadecane (Vinderola Reinheimer 2003).

Results and discussion

Plasmid profiles of *Lactobacillus plantarum* strains

To avoid using similar isolated strains for further analysis, *L. plantarum* strains were differentiated by plasmid profile (Fig. 1). In all, 12 different plasmid profiles were detected out of 16 strains. The *L. plantarum* strains analyzed harbored a large number of plasmids—between 2 and 10—with molecular sizes ranging between approximately 2 and 20 kb. *L. plantarum* strain 66 had the fewest plasmids (2) while *L. plantarum* 22 had the most (10). The largest plasmid was detected in *L. plantarum* 25 (20 kb), whereas the smallest plasmid (3.5 kb) was found in *L. plantarum* 19. Furthermore, no plasmid present in all *L. plantarum* strains was found. These results showed that *L. plantarum* strains sourced from fermented vegetables included a variable number of plasmids of different molecular size, each of which might carry essential genes for industrial traits. Similarly, Ruiz-Barba et al. (1991) also showed that 35 *L. plantarum* strains isolated from different fermented green olive samples harboured large number of plasmids (from 5 to 16) ranging from 2 to 68 kb in size. A similarly wide variety of plasmid profiles in *L. plantarum* strains isolated from fermented olive samples was also reported by Mourad (2007).

Technological features of *Lactobacillus plantarum* isolates

All the *L. plantarum* strains were able to tolerate the tested NaCl concentrations of 4%, 5% and 6.5%. Even at 8% NaCl, all the strains except strain 24 were able to maintain growth. However, in the presence of 9% NaCl, only *L. plantarum* 3 and 22 were able to grow in the medium, indicating that NaCl concentrations of between 8% and 9% exert an inhibitory effect on *L. plantarum* strains. Concentrations of 6% and 8% NaCl are the levels most often used in the processing of fermented vegetables (Buckenhüskes 1993).

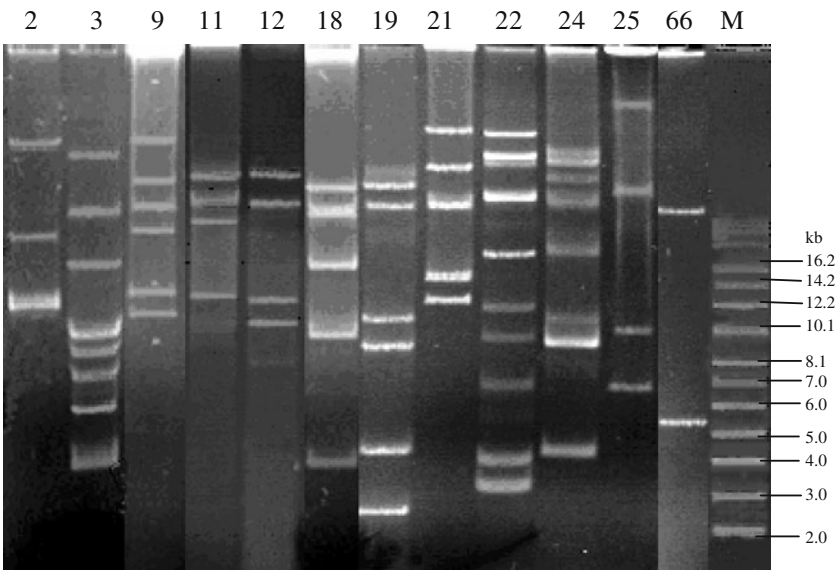

Fig. 1 Plasmid profiles of *Lactobacillus plantarum* strains isolated from fermented vegetables. Lanes: *M* Supercoiled cccDNA marker (Sigma, St. Louis, MO), *2–66* *L. plantarum* strains

Therefore it can be concluded that these *L. plantarum* strains could easily grow in fermentations carried out with high NaCl but, *L. plantarum* 3 and 22 have an advantage over the others due to their high resistance level (Table 1).

Similar proteolytic activity levels were determined for all *L. plantarum* strains and ranged from 0.056 to 0.083 mg/ml tyrosine, which is within the activity range of *L. plantarum* strains isolated from sourdough (Şimşek et al. 2006) and kefir (Yüksekdağ et al. 2004). The maximum proteolytic activity value (0.083 mg/ml tyrosine) was found for *L. plantarum* 2 whereas the minimum value (0.056 mg/ml tyrosine) was seen in *L. plantarum* strains 12 and 19. The proteolytic activity of lactic cultures has proved useful in enhancing the aroma profile of fermented foods by providing some released amino acids that mediate formation of the precursors of aromatic compounds (Buckenhüskes 1993). Consequently, due to their high proteolytic activity, *L. plantarum* strains 2, 9 and 22 should be taken into consideration (Table 1).

The processes of lyophilization and freezing allow the genetic stability of lactic cultures to be maintained, thus preventing the loss of desirable technological properties (Klingberg and Budde 2006). In fact, starter cultures in a lyophilized form have been widely preferred for industrial applications. In the current study, *L. plantarum* strains maintained their viability at approximately similar levels after application of either freezing or lyophilization. However, the freezing process obviously reduces the cell amount of all strains more than lyophilization. Among the strains tested, the highest reduction in viability was obtained with *L. plantarum* 66; strains 12, 19, 21 and 25 were also able to survive at average values of only 82.5% and 91.1% for freezing and lyophilization, respectively. In contrast, *L. plantarum* strains 3 and 18 retained 100% viability and *L. plantarum* strains 2 and 22 grew satisfactorily after both freezing and lyophilization, indicating that these strains could be stored efficiently and used as starter cultures in manufacturing processes. Similarly, G-Allegria et al. (2004) reported that *L. plantarum* strains originating from different sources survived at rates of over 90% after lyophilization and freezing (Table 1).

Survival under conditions simulating the human GI tract

Once LAB have been ingested orally with food, they must first survive transit through the stomach, where the secretion of gastric acid constitutes a primary hurdle to overcome prior to reaching the intestinal tract (Dunne et al. 1999). Therefore, the resistance and tolerance level of any LAB to be used as a putative probiotic strain must be evaluated in vitro against the major components of gastric juice, for instance, low pH, bile, pepsin and pancreatin to mimic the conditions in vivo in the GI tract (Conway et al. 1987; Dunne et al. 1999). The resistance levels to this stimulated gastric juice of the *L. plantarum* strains tested here were evaluated in a solution containing 0.3% (w/v) pepsin and 0.5% (w/v) NaCl at pH 2 and pH 3 separately (Table 2). *L. plantarum* strains maintained viability (58–79%) after 3 h of exposure at pH 3. Some strains showed similar resistance levels, and the strains could be separated into two groups having viabilities of 58–72% and 75–79%, indicating that *L. plantarum* strains isolated from fermented vegetables exhibit different resistance levels to pH and pepsin in the GI tract. In the same solution, *L. plantarum* 11 showed the highest resistance whereas the lowest viability was detected with *L. plantarum* 24 (Table 2). In contrast to resistance at pH 3, at pH 2 *L. plantarum* strains were less viable, indicating that low pH values together with the presence of pepsin and NaCl had a strong inhibitory effect

Table 2 Probiotic characteristics of *Lactobacillus plantarum* strains

L. plantarum strain	Survival in GI tract solution (%)		Bile salt resistance (%)		β-Galactosidase activity	Hydrophobicity (%)
	pH2	pH3	1% bile	4% bile		
2	0	79	103	88	+[a]	54
3	0	79	3	3	−	80
9	0	78	109	90	±	65
11	0	78	105	91	±	50
12	0	75	107	88	±	68
18	18	72	95	87	+	30
19	36	60	89	85	+	33
21	27	58	102	88	+	37
22	21	76	79	63	+	60
24	0	59	62	0	+	31
25	0	60	99	85	+	53
66	20	67	49	40	+	73
Average	9.4	69.1	82.7	65.9		52.0

Data are means of duplicate cultures. *GI* Gastrointestinal

[a] + High activity, ± weak activity, − no activity

on the strains. Only *L. plantarum* strains 18, 19, 21, 22 and 66 were able to survive (viability ranging from 18% to 36%) at this pH. It should be noted that, although the pH of human stomach is 1 during fasting, it ranges from 2 to 4 following a meal. Therefore, the high resistance of *L. plantarum* strains 18, 19, 21, 22 and 66 might allow survival in the low pH of the stomach. In fact, several notable *Lactobacillus* strains have been found to retain viability when exposed to pH values of 2.5 to 4.0, but displayed loss of viability at lower values (Conway et al. 1987; Dunne et al. 1999; Maragkoudakis et al. 2006, 2009).

The effect of bile salts on the viability of *L. plantarum* strains was assessed in both solid and liquid medium at different concentrations (1–7%). The results showed that all the *L. plantarum* strains had high levels of tolerance to bile salts even after exposure to 7% bile salts in solid medium. However, some of the *L. plantarum* strains lost viability after 10 h of incubation in liquid medium with 1% or 4% bile salts (Table 2), indicating that bile salts in liquid medium have a higher inhibitory effect than in solid medium. Accordingly, *L. plantarum* strains 2, 9, 11, 12, 18, 19, 21 and 25 differed from strains 3, 22, 24 and 66 in terms of their high bile salt resistance level (Table 2). Therefore, 62% (8/13) of the *L. plantarum* strains originating from fermented vegetables showed high tolerance against bile salt stress. Bile acids are synthesized in the liver from cholesterol and are secreted from the gall-bladder into the duodenum in conjugated form, so that the concentration in the intestine ranges between 0.3 and 0.5%. Deconjugated forms of bile acids are known to exhibit an antimicrobial effect on Gram-negative as well as Gram-positive bacteria (Dunne et al. 1999). However, most of the *L. plantarum* strains tested here were found to be more resistant to such conditions than some other well known probiotic *Lactobacillus* strains (Conway et al. 1987; Charteris et al. 1998; Maragkoudakis et al. 2006, 2009), suggesting that these strains could survive in the small intestine.

β-Galactosidase activity and antibiotic susceptibility

Out of 13 isolated *L. plantarum* strains, 9 (92%) showed high β-galactosidase activity; 3 strains (*L. plantarum* 9, 11 and 12) had weak activity. In contrast, only one strain, *L. plantarum* 3, had no enzyme activity (Table 2). Therefore, the high β-galactosidase activity of *L. plantarum* strains, as similarly reported by others (Cebeci and Gürakan 2003; Randazzo et al. 2004), could represent a significant metabolic activity in cases of lactose intolerance.

The antibiotic susceptibilities of the isolated *L. plantarum* strains are shown at Table 3. All the *L. plantarum* strains showed a similar resistance pattern against the antibiotics tested (inhibitors of cell wall synthesis and inhibitors of protein synthesis) with some exceptions. *Lactobacillus plantarum* strains were notably susceptible to inhibitors of protein synthesis (4/8). However, the same strains showed resistance to inhibitors of cell wall synthesis with the exception of ampicillin (4/5). Among the strains tested, *L. plantarum* 66 was the most sensitive considering its high susceptibility to the antibiotics used (8/13). Additionally, *L. plantarum* strains showed variable behavior towards penicillin and tetracycline (Table 3). The susceptibility

Table 3 Antibiotic susceptibilities of *Lactobacillus plantarum* strains

L. plantarum strain	Susceptibility to antibiotics[a]												
	P	V	B	A	Pb	T	E	C	R	G	S	K	N
2	S[b]	R	R	S	R	S	S	S	S	R	R	R	R
3	R	R	R	S	R	R	S	S	S	R	R	R	R
9	R	R	R	S	R	S	S	S	S	R	R	R	R
11	R	R	R	S	R	S	S	S	S	R	R	R	R
12	R	R	R	S	R	S	S	S	S	R	R	R	R
18	R	R	R	S	R	S	S	S	S	R	R	R	R
19	R	R	R	S	R	S	S	S	S	R	R	R	R
21	R	R	R	S	R	S	S	S	S	R	R	R	R
22	S	R	R	S	R	R	S	S	S	R	R	R	R
24	R	R	R	S	R	S	S	S	S	R	R	R	R
25	S	R	R	S	R	R	S	S	S	R	R	R	S
66	S	S	S	S	R	S	S	S	S	R	R	R	R

[a] Inhibitors of cell wall synthesis: *P* Penicillin (15 μg), *V* Vancomycin (30 μg); *B* Bacitracin (0.04U), *A* Ampicillin (10 μg), *Pb* Polymyxin B (300 U); inhibitors of protein synthesis: *T* Tetracycline (30 μg), *E* Erythromycin (15 μg), *C* Chloramphenicol (30 μg), *R* Rifampin (5 μg), *G* Gentamicin (10 μg), *S* Streptomycin (30 μg), *K* Kanamycin (30 μg), *N* Neomycin (30 μg)

[b] *R* Resistant, *S* sensitive

results of *L. plantarum* strains were considered to be consistent with minimum inhibitory concentration (MIC) values for antibiotics for *Lactobacillus* strains as reported in the journals SCAN (2002) and EFSA (2005), although the determination methods clearly differed. Recently, Georgieva et al. (2008) tested the resistance to some antibiotics of some *L. plantarum* strains isolated from white brined cheese; similar susceptibility results were obtained as in this study except for ampicillin. Variations in resistance of *Lactobacillus* to penicillin and tetracycline was previously reported by Temmerman et al. (2003) and attributed to the potential transferability of antibiotic resistance determinants. Generally, *Lactobacillus* strains have been known as resistant to aminoglycoside group antibiotics (gentamicin, kanamycin, neomycin, streptomycin) and susceptible to β-lactam (penicillin and ampicillin), Gram-positive spectrum (erythromycin, novobiocin) and broad spectrum (chloramphenicol, rifampin, tetracycline) antibiotics (Charteris et al. 1998; Cebeci and Gürakan 2003; Temmerman et al. 2003; Zhou et al. 2005). Therefore, the *L. plantarum* strains used in this study showed similar susceptibility patterns to those previous findings. One of the required properties for probiotic strains is their safety for human consumption without harboring acquired and transferable antibiotic resistance (Zhou et al. 2005). In this regard, *L. plantarum* 66 can be confirmed as safe for use in animal and human feed. However, for the other strains, a genetic analysis should be carried out to investigate whether resistance to relevant antibiotics is genetically transmissible or not. Nevertheless, intrinsically antibiotic-resistant strains may benefit patients whose normal intestinal microbiota has become unbalanced or greatly reduced in numbers due to the administration of various antimicrobial agents (Zhou et al. 2005).

Hydrophobicity

The isolated *L. plantarum* strains showed different levels of hydrophobicity to *n*-hexadecane, ranging between 30% and 80% (average 52%; Table 1). Hydrophobicity of *L. plantarum* strains 18, 19, 21 and 24 were below average values, and the highest hydrophobicity were measured for *L. plantarum* 3 with 80%, followed by *L. plantarum* 66 with 73%. However, the hydrophobicity of the majority (47%) of *L. plantarum* strains ranged between 53% and 65%, i.e., close to the average value. In most studies, *Lactobacillus* spp. isolates originating from intestinal flora have been shown to possess high hydrophobic properties, which is related to the adhesion ability of these strains to the host (Ouwehand et al. 1999). The hydrophobicity levels of *Lactobacillus* strains including *L. acidophilus*, *L. reuteri*, *L. fermentum* have been measured as ranging between 12% and 68% (Vinderola and Reinheimer 2003) Therefore, all the *L. plantarum* strains tested here, especially 3 and 66, had high hydrophobicity activity, indicating that these strains could adhere to the intestinal mucosa.

Conclusion

In this study, different *L. plantarum* strains were compared for their technological and basic probiotic

characteristics for use as starters for vegetable fermentation. Larger variations were observed in the probiotic properties compared to technological features among *L. plantarum* strains. *Lactobacillus plantarum* 22 was found to be the most efficient strain considering both aspects. However, *L. plantarum* 66 was also an interesting strain, particularly in view of its effective probiotic characteristics, whereas technological properties were optimal for *L. plantarum* 3. Consequently, research using these three strains in different combinations in model fermentation systems is now being carried out to assess their stability and efficiency as starter cultures.

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