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ORIGINAL ARTICLE

In vitro screen of *Lactobacillus plantarum* as probiotic bacteria and their fermented characteristics in soymilk

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Abstract One hundred and two strains of *Lactobacillus plantarum*, all isolated from traditional dairy products of minority nationalities, were evaluated for in vitro probiotic properties, including acid and bile tolerance, aggregation activity, and antibacterial activity. Of these, 12 strains with a high tolerance to simulated gastric juice (pH 2.5, 3 h of incubation) were selected, of which eight of these also showed a good tolerance to bile salt. All selected 12 strains showed a high autoaggregation percentage after being incubated at room temperature for 20 h, and nine of these significantly inhibited growth of the five indicator intestinal pathogens. Six *L. plantarum* strains (IMAU10120, IMAU10156, IMAU40126, IMAU70004, IMAU60042, and IMAU60171) were selected for further evaluation of their fermentation characteristics, sensory quality, and viable counts, both during the fermentation process of soymilk and following a 28-day storage of the soymilk. The six strains had an acid-producing ability at the refrigerated temperature. All of the fermented soymilk samples had a viable count above log 8.45 CFU/ml, and viability in the samples was maintained during storage. Based on both the probiotic properties and fermented characteristics, *L. plantarum* IMAU10120 was the best potential probiotic strain of those tested for the production of fermented functional soymilk.

Keywords *Lactobacillus plantarum* · Probiotics properties · Bile tolerance · Aggregation activity · Viability · Fermented soymilk

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Introduction

Probiotics have become a major focus of lactic acid bacteria (LAB) research over the past 10 years, with most attention focusing on the genera *Lactobacillus* and *Bifidobacterium* (Kailasapathy and Chin 2000). These organisms have been widely reported to exert many beneficial effects, such as activation of the immune system, prevention of cancer cell growth, maintenance of mucosal integrity, and presentation of an antagonistic environment for pathogens (Jin et al. 2011; Kazuhiro and Joseph 2000; Reid et al. 2003; Sanders and Klaenhammer 2001). As a result, the incorporation of these probiotic bacteria into food products with the aim to increase the therapeutic value of these products has become a popular trend (Parvez et al. 2006; Williams 2010).

Interest has been growing in the commercial utilization of *Lactobacillus* strains isolated from traditional, naturally fermented dairy products, which possess health-promoting effects. Research on lactobacilli isolated from such traditional, naturally fermented dairy products reveals a long history of safe use (Holzapfel et al. 2001). In many cases, lactobacilli are also used as starter cultures in industrial and artisanal food fermentation as they contribute to the

conservation, flavor, and texture of the fermented foods. Over 3,432 strains of LAB from traditional, naturally fermented dairy products in China and Mongolia have been isolated, identified by 16S-rDNA analysis, and stored in our laboratory. However, to provide health benefits, *Lactobacillus* strains, which are mostly delivered in a food system, must overcome physical and chemical barriers in the gastrointestinal tract, especially acid and bile stresses, and have antagonistic activity against bacterial pathogens.

Soymilk, a traditional oriental food beverage, is a water extract of soybean that provides a rich yet inexpensive supply of protein and calories. Soymilk can be used as an economical protein beverage when cow milk is not available or is expensive. However, the consumption of soybean as a raw food material has been hindered due to its unfavorable beany flavor (Arai et al. 1996; Scalabrini et al. 1998), the presence of flatulence factors, and the high contents of indigestible alpha-galactosyl oligosaccharides, such as raffinose and stachyose (Tsangalis and Shah 2004). One possible solution to overcoming the off-flavor associated with soy products, such as soy yogurt, is to use LAB exhibiting reductase activity in the fermentation process. The fermentation of soymilk with various organisms, especially LAB, has been reported to eliminate both the beany flavor and flatulence factors, thereby increasing both the acceptability and the nutritional value of the processed product (Jen-Wan et al. 2000; Osaana et al. 2007; Rekha and Vijayalakshmi 2011). Hepatic lipid accumulation-preventing activity, antioxidative, and antimutagenic activities have also been reported. All of these activities are well associated with the selection of LAB (Hsieh and Chou 2006; Kitawaki et al. 2009; Wang et al. 2006), which has led to attempts to achieve fermentation with various LAB.

In the study reported here, 102 *Lactobacillus plantarum* strains isolated in our laboratory from traditional fermented foods were screened for desirable probiotic traits, such as acid and bile tolerance, aggregation activity, and antibacterial activity. The selected *L. plantarum* strains were then used as starter culture for soymilk fermentation, and their fermentation characteristics, sensory quality, and viable counts were evaluated after fermentation and following 28 days of soymilk storage.

Materials and methods

Bacterial strains

One hundred and two strains of *L. plantarum* were grown in MRS broth at 37°C for 18 h. All 16S rDNA sequences of these strains were submitted to GenBank and their GenBank accession numbers are listed in Table 1. The indicator strains of antimicrobial activity included *Escherichia coli* O157 882364, *Salmonella typhimurium* S50333, *Shigella flexneri* CMCC(B)51592, *Staphylococcus aureus* ATCC 1448, and *Listeria monocytogenes* C53-3, which were obtained from our laboratory and cultured in Luria–Bertani (LB) medium, nutrient broth (NB) medium, tryptic soytone broth (TSB), and broth brain–heart infusion agar (BHI), respectively. All strains were subcultured twice prior to being used in the experiments.

Screening of *L. plantarum* for potential probiotic properties

Tolerance to the low pH conditions Cultures of the 102 strains of *L. plantarum* listed in Table 1 were inoculated at 2% in MRS broth (pH 3.0) and grown overnight. All strains were inoculated into phosphate buffered saline (PBS) buffer (pH 3.0) and incubated anaerobically at 37°C for 72 h according to the method described by Conway et al. (1987). The OD value was determined at 600 nm at 0 and 72 h, and $\Delta A_{600nm} \geq 0.500$ was chosen as a standard; the viable plate count method of survival rate was used for further assessments.

Transit tolerance of selected strains Simulated gastrointestinal juice and simulated intestinal juice were prepared according to the method described by Yan et al. (2010). Test cultures (1%) were separately inoculated into simulated gastric juice at pH 2.0 and 2.5, respectively; following mixing for 10 s, the mixtures were incubated anaerobically at 37°C. Gastric transit tolerance was studied by determining total viable counts in gastric juice withdrawn at 0, 1, 2, and 3 h. After 3 h of incubation in gastric juice, 1 ml of culture was inoculated into 9 ml simulated intestinal juice (pH 8.0) and incubated at 37°C anaerobically. The intestinal transit tolerance was studied by determining the total viable counts in intestinal juice withdrawn at 0, 4, 8, 12, and 24 h.

Determination of total viable count and survival rate Viable counts of *L. plantarum* were made using a pour plate method and MRS agar after serial dilution in maximum recovery diluent. A pre-prepared test sample (1 ml) of 10^{-3}, 10^{-4}, and/or 10^{-5} dilution was transferred into a sterile petri dish, in duplicate, and warm (45±1°C) sterile plate count MRS agar (15 ml) was mixed with the inoculum. The plates were incubated anaerobically at 37°C for 48 h, and the colony forming units (CFU) estimated. Survival rate was calculated as: survival rate (%) = $(N_1/ N_0) \times 100\%$, Where N_1 represents the total viable count of probiotic strains after treatment by PBS or simulated gastrointestinal juices and N_0 represents the total viable count of probiotic strains before treatment.

Bile tolerance test The method of Walker and Gilliland (1993) was used to determine the bile tolerance of these

Table 1 Origin of the102 strains of *Lactobacillus plantarum* used in this study

Strains (number)	Origin	Area	Genbank accession number
IMAU10011, 10012, 10013, 10014, 10015, 10016, 10025 (7)	Koumiss	Inner Mongolia	FJ749575, 749576, 749577,749578, 749579, 749580, 749585
IMAU10022, 10023, 10053, 10058, 10062, 10070, 10079, 10114, 10115, 10117, 10118, 10120, 10121, 10124, 10125, 10128, 10140, 10141, 10145, 10155, 10156, 10159, 10160, 10166 (24)	Mixed sample[a]	Inner Mongolia	FJ749582, 749583, 915709, 915714, 915718, 915726, 915735, 915770, 915771, 915773, 915774, 915776, 915777, 915780, 915781, 915784, 915796, 915797, 915801, 915810, 915811, 915814, 915815, 915821
IMAU70004, 70005, 70010, 70023, 70035, 70042, 70080, 70088, 70089, 70090, 70100, 70099, 70087, 70092, 70093, 70094, 70095, 70098, 70101, 70103, 70104, 70105, 70125, 70126, 70164 (25)	Naturally fermented congee	Inner Mongolia	GQ131121, 131122, 131126, 131139, 131151, 131158, 131196, 131204, 131205, 131206, 131216, 131215, 131203, 131208, 131209, 131210, 131211, 131214, 131217, 131219, 131220, 131221, 131241, 131242, 131279
IMAU50045 (1)	Dairy fan	Yun Nan	FJ749445
IMAU40070, 40072, 40082, 40089, 40090, 40091, 40100, 40116, 40117, 40122, 40126 (11)	Fermented Yak milk	Qing Hai	FJ749345, 749347, 749357, 749364, 749365, 749366, 749375, 749387, 749388, 749392, 749395
IMAU40001, 40003, 40005, 40007, 40009, 40010, 40014 (7)	Koumiss	Qing Hai	FJ749720, 749722, 749724, 749726, 749728, 749729, 749733
IMAU30001, 30043, 30055, 30106, 30114, 30110, 30118, 30151, 30159 (9)	Koumiss	Xin Jiang	FJ749604, 749637, 749647, 749677, 749682, 749683, 749685, 749711, 749716
IMAU60026, 60042, 60045, 60047, 60049, 60051, 60055, 60057 (8)	Cattle‘s milk	Tibet	FJ7211392, 211391, 749770, 749772, 749774, 749776, 749779, 749781
IMAU60170, 60171 (2)	Kurut	Tibet	FJ749884, 749885
IMAU20063, 20089 (2)	Mixed sample[a]	Mongolia	FJ640996, 640997
IMAU20009, 20013, 20014, 20015, 20020, 20029 (6)	Koumiss	Mongolia	FJ844939, 844943, 844944, 844945, 844949, 844957

[a] Mixed sample includes cheese, fermented cow milk, natural fermented goat milk, goat milk, yogurt, and cow milk

tested strains. MRS-THIO broth (MRS supplemented with 0.2% sodium thioglycollate) (Kanto, Japan) was inoculated with 10 CFU/ml from overnight cultures. Test cultures were supplemented with 0.3% oxgall (dehydrated fresh bile; Difco, Detroit, MI). All samples were incubated for 24 h at 37°C in a water bath. Growth in control (no bile) and test cultures (0.3% oxgall) was monitored hourly by measuring absorbance at 620 nm using a U-3900 spectrophotometer (Hitachi, Tokyo, Japan) against the corresponding non-inoculated blank samples. Growth curves were plotted, and the analysis was based on the time required for each culture to reach a difference of 0.3 units between the control and test culture. The growth delay in time (hours) between the culture media was considered to be the lag time.

The maximum concentration of bile salts tolerated by the tested strain was determined. MRS broth supplemented with different concentrations of bile salts (0.3, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8%, w/v; B3426, Sigma, St Louis, MI) were used and inoculated with 1% overnight suspensions of the tested strain, and then incubated anaerobically at 37°C for 24 h. Absorbance (620 nm) was measured at 0 and 12 h.

Autoaggregation assays Autoaggregation abilities were measured as described by Collado et al. (2008) as the autoaggregation percentage. Briefly, cells were washed twice with PBS (130 mM sodium chloride, 10 mM sodium phosphate, pH 7.2), resuspended in the same buffer, and adjusted to OD_{660}=0.25±0.05 in order to standardize the number of bacteria (10^7–10^8 CFU/ml). The bacterial suspensions were then incubated at room temperature and monitored at different time intervals (5, 10, 20 h). Autoaggregation percentage was expressed as: $A\% = (A_0 - A_t)/A_0 \times 100\%$, where A_0 represents the absorbance (A_{600nm}) at 0 h and A_t represents the absorbance (A_{600nm}) at 5, 10, and 20 h, respectively.

Coaggregation assays of pathogens with probiotic strains For the coaggregation test, bacterial suspensions were prepared as described for the autoaggregation assay. Equal volumes of cells (500 μl) of various probiotic and pathogen strains were mixed and incubated at room temperature without agitation. The absorbance ($A_{600\ nm}$) of each probiotic and pathogen mixture and for each bacterial suspension alone was measured during the incubation period. Coaggregating ability was expressed as: $[(A_{pat} + A_{probio})/2 - (A_{mix})]/(A_{pat} + A_{probio})/2 \times 100\%$, where A_{pat} and A_{probio} represent the absorbance (A_{600nm}) of each separate bacterial suspension in a control tube, A_{mix}

represents the absorbance (A_{600nm}) of each mixed bacterial/probiotic suspension at the different times tested.

Inhibition of pathogens The methods used for the well diffusion assay of the capacity of the strains to inhibit a representative group of intestinal pathogens were those described by Vinderola et al. (2008) with some modifications. Briefly, 20 μl of MRS agar at 45°C was vigorously mixed with 200 μl overnight culture of the indicator strain and poured into regular microbiological plates. Wells (diameter 8 mm) were punched in the agar layer, and 100 μl of cell-free supernatant, obtained by centrifugation of the cultures at 2,500*g* for 10 min and filter-sterilization through a 0.22-μl pore membrane, was placed in each well.

Fermentation characteristics of the screened *L. plantarum* strains in soymilk

Production of fermented soymilk Soymilk was prepared and fermented according to the procedures described by Wang et al. (2002). The soymilk was first pasteurized at 95°C for 15 min, then cooled to 40°C and inoculated with the screened strains (2×10^7 CFU/ml). After inoculation, the soymilk was incubated at 42°C until fermentation was complete and the pH fell to 4.5. Samples of the fermented soymilk were stored for 28 days at 4°C. Soymilk samples were assayed prior to fermentation, every 3 h during the 24-h fermentation process, and on days 0, 7, 14, 21, and 28 of storage. The trial was carried out in triplicate.

Fermentation characteristics The pH values were measured using a pHSJ-3 F pH meter (Leici, Shanghai, China). The titratable acidity (TA) was determined with 0.1N NaOH using a 0.5% phenolphthalein as indicator. The viable counts of the screened *L. plantarum* strains were enumerated according to the description of Tharmaraj and Shah (2003).

Sensory evaluation Descriptive sensory analysis was performed by a panel of ten trained panelists on days 0, 7, 14, and 21 o the storage period. Panel members discussed and agreed upon the definitions and how to qualify the attributes on the chosen scale. Panelists and test rooms of sensory analysis were according to international standards (ISO-8586-1 1993) and (ISO-8589 1988), respectively. A beaker (100 ml) filled with the set-type fermented soymilk was used for evaluation with a tablespoon, and the testers rinsed their mouths with water between consecutive samples. The characteristics evaluated included appearance (soy whey-separation, firmness, shape-maintenance, surface-smoothness), texture (hardness, consistency, flatness), flavor (soy flavor, acid flavor, cooking flavor, and global flavor), taste (soy taste, acid taste, bitter taste, astringency, greasy texture, and global taste) (Buono et al. 1990; Macedo et al. 1999; Salvador and Fiszman 2004). Each subitem was divided into five grades (five scores: 1= strongly attractive, 5=strongly unappealing), and item and total scores were calculated and analyzed using SPSS software (SPSS, Chicago, IL) to determine significance.

Statistical analysis

Statistical analysis of the data was performed using SAS ver. 7.0 (SAS Institute, Cary, NC). The comparisons of differences between the means of the treatments were tested by analysis of variance (ANOVA) at a significance level of $P<0.05$.

Results and discussion

Screening of *L. plantarum* strains with desired potential probiotic properties

The screening results showed that 30 strains of *L. plantarum* grew well ($\Delta A_{600nm}\geq0.500$) at the acidic condition of pH 3.0 (data not shown); this pH was selected for further analysis of transit tolerance. These 30 strains were then screened for a high tolerance to the simulated gastric juice (pH 2.5, 3-h incubation); 12 strains met the criterion of a survival rate >1% (Table 2). Although most of the tested strains could grow in an acidic environment, there was a large variation in growth rate when they were cultured in artificial gastric juice at pH 2.5 for 3 h (Table 2).

Of the 12 *L. plantarum* strains incubated in the simulated gastric juice (pH 2.0), five (IMAU10120, IMAU20029, IMAU70004, IMAU60171, IMAU60042) exhibited fairly good acid tolerance and were able to survive (range 7.3–3.5 log CFU/ml). Incubation in the simulated intestinal juice up to 24 h also did not significantly affect the viability of these five strains (Table 3). IMAU10156 and IMAU40126 survived with viable counts of about 3.0 log CFU/ml in the simulated intestinal juice for 4 h, but not for 8 h; the remaining five strains did not tolerate incubation in the simulated gastric juice and intestinal transit.

The bile tolerance of the 12 screened *L. plantarum* strains in the form of lag time is given in Table 4, and the tolerance to different concentrations of bile salts is shown in Table 5. According to the standards established by Chateau et al. (1994), IMAU70089, IMAU20029, IMAU30055, IMAU60171, IMAU70004, IMAU10120, and IMAU40126 were assessed as bile-tolerant strains, IMAU70042, IMAU60042, and IMAU10156 as weakly tolerant strains, and IMAU10058 and IMAU40005 as

Table 2 Survival of 12 strains of *L. plantarum* in artificial gastrointestinal juice at pH 2.5

Strains	Tolerance to simulated gastric juice at pH 2.5 (log CFU/ml)		Survival rate (%)
	0 h	3 h	
IMAU70089	8.889±0.004	9.002±0.015	129.68 a
IMAU20029	8.961±0.017	8.916±0.004	90.16 b
IMAU30055	8.787±0.055	8.642±0.056	71.54 c
IMAU60171	9.366±0.017	9.079±0.005	51.61 d
IMAU70004	9.184±0.028	8.838±0.027	45.10 d,e
IMAU10120	9.242±0.061	8.823±0.005	37.89 e,f
IMAU40126	8.993±0.062	8.560±0.059	36.87 f
IMAU70042	9.085±0.040	8.400±0.013	26.23 g
IMAU60042	9.073±0.044	8.473±0.008	25.06 g
IMAU10156	8.938±0.057	8.239±0.088	21.72 g
IMAU10058	9.467±0.054	8.331±0.043	7.31 h
IMAU40005	9.163±0.046	7.728±0.006	3.66 h

CFU, Colony-forming units

Data are presented as the mean ± standard deviation (SD) from the mean of triplicate determinations. Values within a column followed by different lowercase letters are signficantly different at $P<0.05$

sensitive strains (Table 4). To simulate intestinal conditions, a broader range of bile concentrations was then tested, which involved the adaptation to a series of high concentrations of bile salt (range 0.3–1.8%). The highest tolerance level of IMAU70089, IMAU60171, IMAU70004, IMAU70042, IMAU10156, IMAU40126, IMAU10120, and IMAU60042 to bile salts was 1.8% (Table 5).

Despite the simplicity of the technique, the results of this experiment scientifically validated the bile tolerance capacity of IMAU70089, IMAU60171, IMAU70004, IMAU10156, IMAU40126, IMAU10120, IMAU60042, and IMAU70042. The other four strains tested were considered to be sensitive to bile (Tables 4, 5).

The results of the autoaggregation assays are shown in Table 6. The highest autoaggregation was observed in IMAU40126, whose aggregation percentage reached 53.2% following incubation at room temperature for 20 h. IMAU10120, IMAU40005, and IMAU10058 also showed a high autoaggregation percentage after incubation, with aggregation percentages of 46.4, 45.8, and 30.5%, respectively. The remaining strains tested also showed a high autoaggregation percentage following incubation, with aggregation percentages of >20%.

All 12 tested *L. plantarum* strains showed some coaggregation properties with pathogens (Table 7); however, this property was also strain specific and the degree gradually increased with time. As a result, all 12 of the strains tested in this experiment showed coaggregation abilities with the pathogens tested, but the coaggregation percentages depended on the specific *L. plantarum* strain, the specific pathogen strain, and the incubation time, similar to the results reported by Collado et al. (2007). All

Table 3 Viable count of 12 strains of *L. plantarum* artificial gastrointestinal juice at pH 2 and artificial intestinal juice at pH 8

Strains	Tolerance to simulated gastric juice at pH 2.0 (log CFU/ml)				Tolerance to simulated digestive juice at pH 8.0 (log CFU/ml)				
	0 h	1 h	2 h	3 h	0 h	4 h	8 h	12 h	24 h
IMAU70089	9.097±0.045	6.342±0.580	4.702±0206	3.914±0299	3.914±0299	3.819±0262	4.132±0157	3.709±0066	4.380±0.451
IMAU20029	9.386±0.009	3.763±0.021	5.731±0.057	4.870±0.242	4.870±0.242	5.748±0.000	5.744±0.143	5.770±0.042	6.115±0.163
IMAU30055	9.239±0.034	5.322±0.000	2.952±0.069	5.869±0.033	5.869±0.033	3.218±0.147	5.835±0.040	5.809±0.033	3.545±0.031
IMAU60171	9.468±0.029	6.704±0.016	5.241±0.053	5.720±0.082	5.720±0.082	5.172±0.082	5.264±0.213	5.638±0.035	7.267±0.041
IMAU70004	9.0470.008	6.358±0.016	6.018±0.038	5.612±0.030	5.612±0.030	5.343±0.125	5.203±0.038	5.255±0.000	4.809±0.086
IMAU10120	9.275±0.014	6.213±0.017	4.739±0.056	2.827±0.181	2.827±0.181	3.102±0.144	0.000±0.000	—	—
IMAU40126	9.460±0.045	6.827±0.181	3.278±0.281	4.055±0.078	4.055±0.078	3.562±0.025	0.000±0.000	—	—
IMAU70042	9.287±0.044	5.961±0.010	3.633±0.014	0.000±0.000	—	—	—	—	—
IMAU60042	9.002±0.028	6.085±0.050	3.840±0.097	0.000±0.000	—	—	—	—	—
IMAU10156	8.949±0.021	4.552±0.080	0.000±0.000	—	—	—	—	—	—
IMAU10058	9.258±0.007	3.395±0.074	0.000±0.000	—	—	—	—	—	—
IMAU40005	9.045±0.006	2.841±0.088	0.000±0.000	—	—	—	—	—	—

Data are presented as the mean ± SD from the mean of triplicate determinations

Table 4 Bile tolerance, measured as lag time, of 12 strains of *L. plantarum* to 0.3% bile salt in medium*

Strains	Absorbance at 620 nm measured per hour		
	Not-supplemented with oxgall	Supplemented with 0.3% (w/v) oxgall	Lag time (h)
IMAU70089	2.75±0.08	3.07±0.12	0.32±0.07 a
IMAU20029	4.18±0.04	4.55±0.26	0.37±0.10 a,b
IMAU30055	3.75±0.13	4.20±0.31	0.45±0.01 b,c
IMAU60171	3.37±0.34	3.86±0.27	0.49±0.05 c,d
IMAU70004	3.46±0.11	4.02±0.25	0.56±0.06 c,d
IMAU10120	3.77±0.44	4.34±0.20	0.57±0.02 d
IMAU40126	4.44±0.07	5.01±0.41	0.57±0.05 d
IMAU70042	3.12±0.01	3.84±0.28	0.72±0.10 e
IMAU60042	3.92±0.34	4.65±0.24	0.73±0.11 e
IMAU10156	3.30±0.02	4.12±0.23	0.82±0.09 e
IMAU10058	3.79±0.24	4.88±0.17	1.09±0.03 f
IMAU40005	3.29±0.02	5.17±0.14	1.89±0.05 g

Data are presented as the mean ± SD from the mean of triplicate determinations. Values within a column followed by different lowercase letters are signficantly different at $P<0.05$

Table 6 Autoaggregation activity of 12 strains of *L. plantarum*

Strains	Incubation time		
	5 h	10 h	20 h
IMAU40126	21.3±2.8	26.5±0.9	53.2±2.2 a
IMAU10120	16.9±1.5	22.7±2.0	46.4±1.3 b
IMAU40005	17.7±3.9	21.6±1.4	45.8±0.4 b
IMAU10058	11.6±4.1	17.5±4.7	30.5±4.6 c
IMAU60171	15.9±0.6	21.4±1.5	29.6±2.2 c
IMAU20029	14.6±0.4	20.8±1.6	27.9±1.3 c,d
IMAU30055	14.4±3.6	19.5±1.7	27.3±0.9 c,d
IMAU10156	13.4±0.5	17.6±2.6	27.0±0.5 c,d,e
IMAU60042	17.5±0.8	18.2±1.3	25.1±1.2 d,e,f
IMAU70042	12.7±0.5	12.7±0.5	23.4±1.1 e,f
IMAU70004	13.1±0.5	16.1±0.8	22.4±2.9 f
IMAU70089	15.1±0.7	18.8±0.8	22.2±1.7 f

Data are presented as the mean ± SD from the mean of triplicate determinations. Values within a column followed by different lowercase letters are signficantly different at $P<0.05$

tested strains showed high coaggregation percentages with *Staphylococcus aureus*, probably due to its morphology. In general, probiotic strains IMAU40005, IMAU40126, and IMAU10120 showed the best coaggregation properties with pathogens.

The inhibition the 12 tested *L. plantarum* strains to five food spoilage and pathogenic bacteria is shown in Table 8. The cell-free culture supernatants of IMAU70004, IMAU70042, IMAU40126, IMAU10156, IMAU70089, IMAU60171, IMAU40005 IMAU10120, and IMAU60042 in MRS broth was significantly inhibitory to both Gram-positive (*Listeria monocytogenes* C53-3, *S. aureus* ATCC 1448) and Gram-negative bacteria (*Escherichia coli* O157 882364, *Shigella flexneri* CMCC(B)51592, *Salmonella typhimurium* S50333). At the same time, the results indicated that different strains of *L. plantarum* possessed different degrees of antimicrobial activity, even these high acid-tolerant strains, likely due to the production of bacteriocin in the antimicrobial substances.

Table 5 Bile tolerance, measured as change in absorbance, of 12 of strains of *L. plantarum* to different concentrations of bile salts

Strains	Absorbance at 620 nm								
	Concentrations of bile salts (%)								
	0.3	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8
IMAU70089	2.038±0.010	2.057±0.031	2.242±0.003	2.285±0.021	2.286±0.006	2.206±0.026	1.981±0.053	1.844±0.132	1.770±0.054
IMAU60171	1.645±0.125	1.746±0.045	1.844±0.014	1.383±0.537	1.639±0.011	1.431±0.038	1.400±0.037	1.401±0.029	1.462±0.083
IMAU70004	1.276±0.016	1.384±0.038	1.303±0.018	1.283±0.005	1.232±0.015	1.083±0.016	1.101±0.013	1.141±0.013	1.280±0.018
IMAU70042	1.407±0.042	1.295±0.044	1.334±0.014	1.294±0.001	1.301±0.010	1.100±0.021	1.082±0.016	1.110±0.005	1.275±0.013
IMAU10156	1.151±0.056	1.201±0.018	1.129±0.049	1.114±0.066	1.119±0.033	0.980±0.024	0.975±0.012	0.984±0.032	1.124±0.017
IMAU40126	1.324±0.060	1.363±0.116	1.262±0.010	1.260±0.167	1.277±0.078	0.992±0.157	1.103±0.145	1.144±0.083	1.114±0.206
IMAU10120	1.192±0.028	1.277±0.027	1.207±0.050	1.192±0.042	1.172±0.049	1.021±0.062	1.027±0.049	1.013±0.023	1.107±0.011
IMAU60042	1.362±0.017	1.429±0.061	1.276±0.064	1.134±0.017	1.132±0.091	1.033±0.019	1.029±0.023	0.984±0.016	1.031±0.048
IMAU20029	1.447±0.020	1.205±0.030	1.264±0.007	1.280±0.003	1.173±0.017	1.174±0.022	0.960±0.084	0.973±0.003	0.908±0.114
IMAU10058	1.214±0.005	1.063±0.034	1.111±0.008	1.050±0.015	1.069±0.023	1.026±0.006	0.886±0.008	0.885±0.007	0.901±0.009
IMAU30055	1.149±0.037	1.188±0.028	1.030±0.020	1.000±0.025	0.967±0.032	0.678±0.033	0.664±0.029	0.579±0.090	0.626±0.038
IMAU40005	1.072±0.043	0.720±0.010	0.809±0.007	0.746±0.028	0.712±0.048	0.476±0.077	0.410±0.073	0.387±0.034	0.622±0.039

Data are presented as the mean ± SD from the mean of triplicate determinations

Table 7 Coaggregation percentage of 12 strains of *L. plantarum* against five pathogens

Strains	*Listeria monocytogenes* C53-3	*Staphylococcus aureus* AC1.2465	*Shigella flexneri* CMCC(B)51592	*Salmonella typhimurium* S50333	*Escherichia coli* O157 882364
IMAU40005	34.5±2.0 a	40.3±2.9 b,c	38.0±2.3 a,b	36.9±0.7 a	35.1±0.8 b
IMAU40126	32.1±0.9 a,b	39.9±1.3 b,c	39.4±4.6 a	37.4±1.1 a	38.7±1.4 a
IMAU10120	29.7±3.3 b	38.2±1.2 c	34.8±0.4 b,c	34.1±1.3 b	31.6±0.9 b,c
IMAU10156	25.5±0.9 c,d	40.9±0.5 b,c	30.9±0.4 c,d,e	28.9±2.8 d,e	27.2±2.8 e
IMAU70089	22.5±1.0 e	40.9±1.2 b,c	24.7±2.2 f,g	28.3±1.4 e	20.5±2.3 g
IMAU60171	26.8±1.7 c	41.3±2.3 b,c	23.7±1.1 f,g	32.2±1.3 b,c	31.2±1.0 c,d
IMAU70004	18.2±0.6 f	38.9±0.6 b,c	21.8±0.5 g	21.6±0.2 g	19.6±1.6 f,g
IMAU20029	21.8±0.9 e	42.2±1.4 b	26.5±2.5 f,g	24.4±0.9 f	23.0±0.6 f
IMAU60042	22.6±0.5 e	39.7±3.6 b,c	22.0±1.1 g	23.5±0.4 f,g	19.2±0.9 g
IMAU70042	21.3±1.6 e	41.3±0.9 b,c	24.1±3.7 f	27.0±2.0 e	27.5±5.2 d,e
IMAU10058	25.9±0.4 c,d	46.1±2.1 a	33.2±1.9 c,d	31.1±1.3 c,d	30.5±2.0 c,d,e
IMAU30055	23.8±0.9 d,e	49.1±2.1 a	28.1±1.8 e,f	28.2±2.4 e	30.3±0.7 c,d,e

Data are presented as the mean ± SD from the mean of triplicate determinations. Values within a column followed by different lowercase letters are signficantly different at $P<0.05$

Lactobacillus plantarum IMAU70004 demonstrated the highest antagonistic activity against all foodborne pathogens (Table 8), with *L. plantarum* IMAU70042, IMAU40126, IMAU10156, and IMAU70089 showing a gradual lower degree of antibacterial ability against all five indicator strains. In contrast, *L. plantarum* IMAU10058 showed no antibacterial activity to all five pathogens. In particular, *L. plantarum* IMAU20029 had no inhibitory zone against *L. monocytogenes* C53-3 and IMAU30055 had no inhibitory zone against *S. flexneri* CMCC(B)51592 and *S. aureus* ATCC 1448.

Based on the probiotic properties determined in these studies, six strains of *L. plantarum*, namely, IMAU10120, IMAU10156, IMAU40126, IMAU70004, IMAU60042, and IMAU60171, were selected for the fermentation study in soymilk.

Fermentation and storage characteristics of six strains of *L. plantarum* in soymilk

The changes in the pH and TA counts of soymilk inoculated with six strains of *L. plantarum* during fermentation are shown in Fig. 1. Soymilk was found to support the growth of all six of these *L. plantarum* strains. Significant increases in the TA and decreases in the pH in soymilk during fermentation were observed with all six *L. plantarum* strains. Strains IMAU10120 and IMAU10156 required 15 and 13.5 h, respectively, to ferment the soymilk to a final pH

Table 8 Antibacterial activity, measured by the well-diffusion assay, of 12 strains of *L. plantarum* against five pathogens[a]

Strains	*Listeria monocytogenes* C53-3	*Salmonella typhimurium* S50333	*Escherichia coli* O157 882364	*Shigella flexneri* CMCC(B)51592	*Staphylococcus aureus* AC1.2465
IMAU70004	++	+++	+++	+++	++
IMAU70042	++	++	++	++	++
IMAU40126	++	+	++	++	++
IMAU10156	+	+	++	++	+
IMAU70089	++	++	+	+	+
IMAU60171	+	+	±	+	+
IMAU40005	+	+	+	±	+
IMAU10120	±	±	+	±	±
IMAU60042	±	+	±	±	±
IMAU20029	−	±	+	+	±
IMAU30055	±	±	±	−	−
IMAU10058	−	−	−	−	−

[a] Antibacterial zone = diffusion diameter – pH control diameter: −, ≤0 mm ; ±, 0–4 mm; +, 4–8 mm ; ++, 8–12 mm; +++, >12 mm

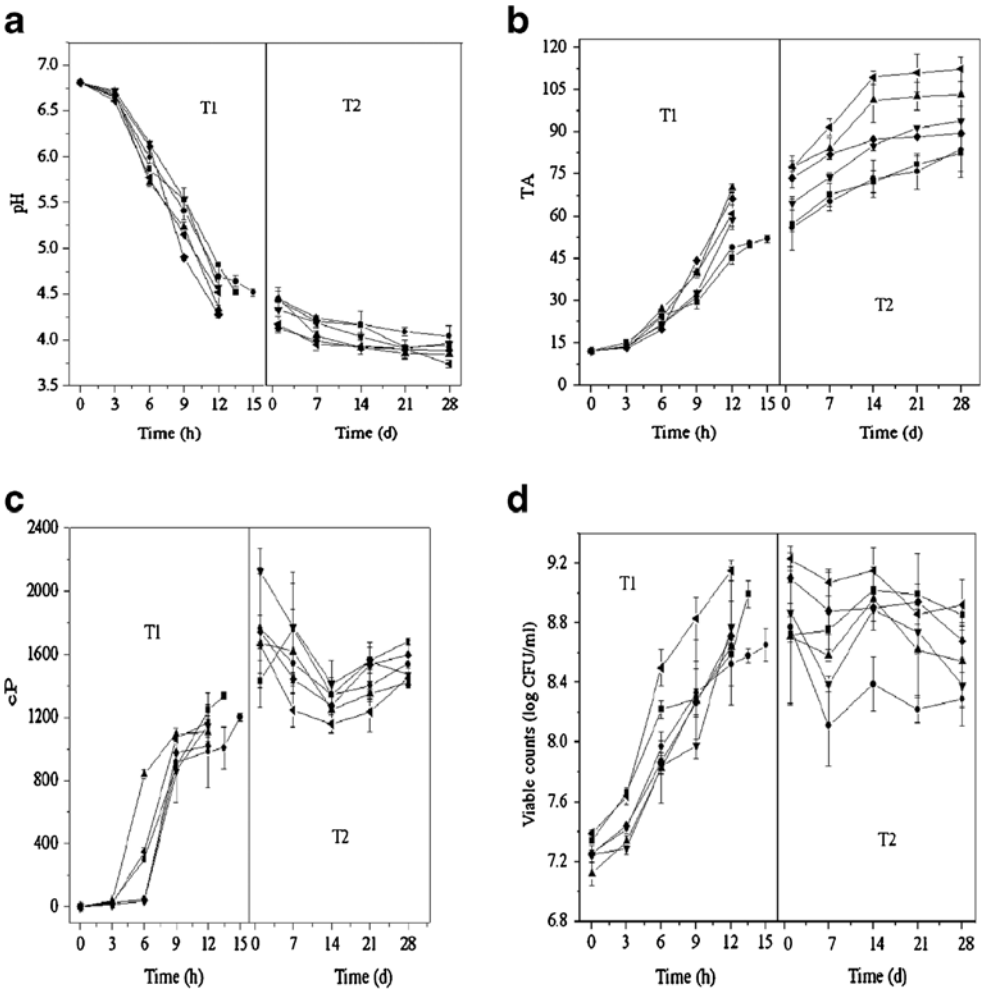

Fig. 1 The fermentation characteristics of six strains of *Lactobacillus plantarum* in soymilk. *T1* Time-line of fermentation, *T2* time-line of storage. The value of each strain is given in the presence of IMAU10156 (*filled square*), IMAU10120 (*filled circle*), IMAU40126 (*upright filled triangle*), IMAU70004 (*inverted filled triangle*), IMAU60042 (*filled diamond*), IMAU60171 (*filled inverted arrowhead*). **a** pH value, **b** titratable acidity (*TA*), **c** viscosity (*cP*), **d** viable count (*log CFU/ml*)

of 4.5; the other four strains tested required 12 h to reach pH 4.5. The final TA of soymilk reached 49.85 °T and 52.01 °T for IMAU10156 and IMAU10120, respectively, while the TA values of the other four strains ranged from 58.74 to 70.06 °T. After storage for 28 days, the TA values of all samples increased to 80–110 °T, thereby demonstrating that these six *L. plantarum* strains had acid-producing ability at the refrigerated temperature.

The changes in the viscosity of the soymilk during fermentation are shown in Fig. 1c. During the fermentation period, the viscosity of soymilk markedly increased with increasing fermentation time, reaching as high as 1,000–1,400 cP at the end of the fermentation period. However, the change in viscosity during the storage period was not significant.

Gradual increases in the viable counts during fermentation were observed (Fig. 1d), and there was no significant difference in the viable counts among the samples at the end of the fermentation. There were also no dramatic differences in the viable counts of all strains in the fermented soymilk samples during storage (Fig. 1d). There was a general trend towards a slight decrease in the number of viable bacteria between storage day 7 and day 21. The viable count of IMAU10120 increased to log 8.51 CFU/ml at the end of the fermentation period and was maintained at log 8.31 CFU/ml at the end of the storage period.

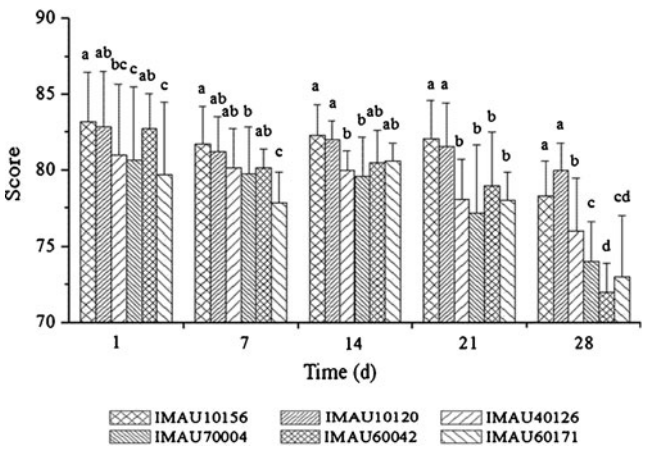

Fig. 2 Sensory evaluation of soymilk after fermentation and during storage. Columns with *different lowercase letters* are significantly different at $P<0.05$

IMAU60171 reached log 9.15 CFU/ml after fermentation and maintained viability at log 8.93 CFU/ml at the end of the storage period, which was the highest recorded count among all strains.

An administered strain of *Bifidobacterium breve* strain Yakult was capable of growing in soymilk with no additives up to a level of 10^9 CFU/ml. During storage of the fermented soymilk at 10°C for 20 days, viable counts of this strain did not change (Shimakawa et al. 2003). This result is similar to our results of fermented soymilk by *L. plantarum*.

To be able to exert its health benefits in vivo, probiotics must be able to enter the intestinal tract alive, which requires them to retain their viability in the food during shelf-life and after consumption and to resist the acidic conditions of the stomach as well as the bile salts in the intestine (Kailasapathy and Rybka 1997). Therefore, a viable cell count of $>10^6$–10^7 CFU/g is necessary to ensure that a sufficient "daily dose" of 10^6–10^9 viable bacteria are supplied (Lee and Salminen 1995). In our experiment, all of the fermented soymilk samples had a viable count of more than log 8.45 CFU/ml and maintained their viability in the samples during storage.

The sensory evaluations of taste were made during the storage period at 1, 7, 14, 21, and 28 days (Fig. 2). The scores of *L. plantarum* IMAU10156 and IMAU10120 were better than those of the other four strains, especially at 28 days of storage, in terms of stable taste and flavor. Although the fermented characteristics of soymilk by *L. plantarum* IMAU10120 were not the best, the taste and flavor scores were favorable, possibly because of the objectionable beany flavor and taste in the other fermented soymilks (low scores). According to Chun et al. (2008), the most important factor for soy yoghurt quality is taste (about 71% of respondents); as such, *L. plantarum* IMAU10120 can be considered to be the most suitable strain for soy yoghurt production.

Conclusions

Of the 102 different strains tested, *L. plantarum* IMAU10120 was the most suitable potential probiotic strain for novel health-promoting fermented products after both the evaluation of in vitro probiotic properties and fermented characteristics. Therefore, these strains will be used in further in vivo probiotic studies in our laboratory to develop probiotic fermented soymilk products.

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