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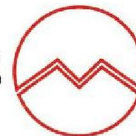

Probiotic Potential of Lactic Acid Bacteria Isolated from Mulberry Silage

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ABSTRACT: Lactic acid bacteria are the most important bacteria that have been used as probiotic in food and feed industries. Due to their beneficial probiotic properties, search for new lactic acid bacterial strains which are more tolerate to the stress conditions of the GIT, and have better probiotic properties than existing strains is still continued. In the present study, a total of 50 isolates were isolated from mulberry (*Morus Alba*) silage as a potential source for lactic acid bacteria. Based on the initial identification using catalase test, gram staining and colony and cell morphology, 38 isolates which were most probably lactic acid bacteria were selected for *in vitro* acid and bile tolerance tests. Of the 38 isolates, 34 were acid tolerance and 21 were bile tolerance. Identification of 10 selected isolates, which exhibited better acid and bile tolerance than the others, using 16S rRNA gene sequence analysis showed that all 10 isolates belonged to the genus *Lactobacillus* including one *L. pentosus*, two *L. farraginis*, two *L. brevis* and five *L. acidipiscis*. Results of studies on reduction of pH in the growth medium and organic acid production profiles of the strains revealed that four selected *Lactobacillus* strains (one strain from each species, namely *L. farraginis* ITA22, *L. pentosus* ITA23, *L. brevis* ITA33 and *L. acidipiscis* ITA44) reduced the pH of their growth medium to the levels of 3.2 to 4.1 during 24 h of incubation by production of organic acids, mainly lactic acid (production of 187.27 to 433.41 mM) and acetic acid (production of 86.79 to 106.21 mM). Generally, the four isolated *Lactobacillus* strains showed good tolerance to acid and bile salts, so they would probably be able to survive in the GIT, and they could be considered as potential probiotic candidates for humans and animals. They produced considerable amounts of organic acids, which could be a positive point toward their antagonistic activity against pathogenic strains. However, further studies are needed to investigate their probiotic properties including antimicrobial activity.
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1. INTRODUCTION

The probiotic concept has been defined by Fuller (1989) as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance”. Lactic acid bacteria (LAB) are Gram-positive bacteria (Fooks *et al.*, 1999) which ferment carbohydrates into energy and lactic acid (Jay, 2000). They are the most important bacteria that have been used as probiotic in food and feed industries (Metchnikoff *et al.*, 1908; Collins *et al.*, 1998; Schrezenmeir *et al.*, 2001). Lactic acid bacteria are known to have probiotic properties such as cholesterol reduction (Noh *et al.*, 1997), anticancer (Choi *et al.*, 2006), antioxidant activity (Ahotupa *et al.*, 1996), and antimicrobial activity by production of antimicrobial substances, mainly organic acids, bacteriocins, and hydrogen peroxide (Caplice *et al.*, 1999). However, to release its probiotic properties, an orally administered probiotic strain has to be able to survive in the gastro-intestinal tract (GIT) of the host by tolerate GIT’s stress conditions, especially acidic pH and presence of bile salts (Ronka *et al.*, 2003). Because of their beneficial properties there is a continues search for new probiotic strains which are more tolerate in the GIT, with better probiotic properties than existing ones.

Mulberry (*Morus alba*) is a genus of flowering plants in the family Moraceae, growing wild or under cultivation in many temperate world regions. It has high edible biomass yield of 16-18 tons dry matter/ha/year, high crude protein content (15-25%) and high *in vitro* dry matter digestibility (75-85%). Hence, mulberry is considered as a good source for feeding and supplementing ruminants (Ojeda *et al.*, 2002). This plant has three months cutting intervals, so conserving it as silage is a good

way to avoid wasting of surplus in the rainy season. Many potential probiotic strains have been isolated from different sources such as fermented animal-origin and plant-origin sources (Jamuna *et al.*, 2004), suggesting that mulberry silage also could be considered as a potential plant-origin source for isolation of potential probiotic strains.

The aim of the present study was to isolate and identify some LAB from mulberry silage and characterize their survivability in the GIT. Reduction in the pH of growth medium and organic acid production by the selected isolates was also investigated.

2. MATERIAL AND METHODS

Isolation of LAB

Lactic acid bacteria were isolated from locally prepared Mulberry silage. To transfer the bacteria from the solid sample into the solvent, 10 gram of silage was dissolved into 100 ml of phosphate buffer saline (PBS, 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in 1 l distilled water, pH 7.2) and shaken at 200 rpm for 10 min at room temperature. Three replicate samples were prepared and from each, tenfold serial dilutions (up to 10^{-7}) were prepared into dilution tubes containing PBS. Of each dilution, 100 µl were spread-plated on de Man, Rogosa and Sharpe (MRS) agar medium (Merck, Germany). Plates were anaerobically incubated at 37°C for 48h. After incubation, several colonies which show typical morphology of LAB were selected from each plate, and were purified by three times sub-culturing on MRS agar plates. The pure isolates were then stored in 20% (v/v) glycerol at -80°C for future analysis.

Catalase test and Gram staining were used to initial identification of the isolates. For Catalase test, one drop of 3% hydrogen peroxide (Sigma, USA) was dropped on the three randomly chosen single colonies of each isolate on MRS agar plate. Bubble forming on the colonies indicates that the isolate is catalase positive and not LAB. However, absence of gas bubbles indicated a negative reaction (Smibert, 1974). Since LAB are Gram positive, the isolates were tested for Gram stain to increase the possibility of LAB in the selected samples. Only catalase negative and Gram positive isolates were selected for characterization of their survival in the GIT (Kandler *et al.*, 1986; Schillinger *et al.*, 1987).

In vitro survival characterization of the isolates

Acid tolerance test

Tolerance to acidity was tested using evaluation of the growth rate of the isolates after exposure to pH 3 (acidic condition) and pH 7.2 (control) for 3 h. The method of Ehrmann *et al.* (2002) was followed with modifications. From overnight culture of each isolate, 100 µl was inoculated into 10 ml of normal (pH 7.2, control) or acidic PBS (adjusted to pH 3 using 5 M hydrochloric acid), and anaerobically incubated at 37℃ for 3 h. Subsequently, 100 µl of each sample was cultivated in the normal MRS broth and anaerobically incubated at 37℃ for 24 h. After that, the growth rates of the tested isolates in MRS broth were determined by reading the absorbance at 620 nm using a spectrophotometer (Barnstead International, USA).

Bile tolerance test

Bile tolerance of the isolates was tested using their growth rate in 0.3% oxgall (Sigma, USA). The method of Jacobsen *et al.* (1999) was followed with modifications. From overnight culture of each isolate, 100 µl was inoculated into 10 ml of MRS broth (control) or MRS broth containing 0.3% oxgall, and incubated anaerobically at 37°C for 4 hours. After that, for the growth rate determination, the absorbances of the samples were read at 620 nm using a spectrophotometer (Barnstead International, USA).

Identification of LAB

DNA from overnight culture of each isolate was extracted using blood and tissue DNA extraction kit from (QIAGEN, Germany) according to the manufacturer's instructions. The PCR amplifications of 16S rRNA genes were carried out using a GeneAmp 9600 PCR system (Perkin-Elmer, US) with the 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') as the forward and reverse primers, respectively. The PCR reaction was performed on a total volume of 50 µl using the *i-StarTaq*™ *DNA Polymerase* kit (*iNtRON* Biotechnology, Korea). Each reaction included 1µl *i-StarTaq*™ *DNA Polymerase* (5 u/µl), 1 µl of each Primer (10 pmol/µl), 5 µl PCR buffer, 5µl dNTP, 2 µl DNA samples and 35 µl deionised water. The PCR reaction was carried out with the following profile: initial hold at 94 °C for 5 minutes, 40 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 40s, extension at 72°C for 2 min, and final extension period at 72 °C for 5 min. The PCR products were purified using PCR purification kit (*iNtRON* Biotechnology,Korea), and forward and reverse DNA were sequenced (1st base Co., Malaysia). Forward and reverse sequences of each isolate were aligned using Bioedit software, version 7.0.9.0 (Hall, 1999), and approximately 1400 bp segment of the 16S rRNA gene of the isolates was compared to strains in the National Center for Biotechnology Information (NCBI) Blast Library (http://blast.ncbi.nlm.nih.gov).

A phylogenetic tree was conducted based on the 16S rRNA gene sequences analysis. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). The analysis involved 23 nucleotide sequences. *Escherichia coli* has been used as outgroup. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The tree was obtained using the Close-Neighbor-Interchange algorithm (Nei *et al.*, 2000) with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree is drawn to scale with branch lengths calculated using the average pathway method (Nei and Kumar, 2000) and in the units of the number of changes over the whole sequence. All positions containing gaps and missing data were eliminated. There were a total of 1300 positions in the final dataset.

Reduction of pH of the growth medium

Growth kinetics with the corresponding pH reduction of the growth medium by four selected LAB strains were determined. For this, 100 µl of overnight culture of each strain was inoculated into 10 ml of MRS broth and incubated anaerobically at 37°C for 2, 4, 8, 12 and 24 h. After that, the growth of the strains in MRS broth were determined by reading the absorbance at 620 nm using a spectrophotometer (Barnstead International, USA), and at the same time intervals, pH of the samples were measured using a pH meter (Comlab, UK).

Organic acid production profile

For determination of volatile fatty acids (VFA) and non-VFA production by the isolated LAB, 4 ml of overnight culture of each isolate in MRS broth were centrifuged in 1500 × *g* for 10 min at room temperature. Then 3 ml of supernatant fluid were collected into 15 ml centrifuge tube and 600 µl of 24% (v/v) metaphosphoric acid was added. The samples were kept for 24 h at room temperature. The samples were then centrifuged in 1500 × *g* for 20 min at room temperature and 0.5 ml of supernatant with 0.5 ml of internal standard (20 mM 4-methyl valeric acid) were transferred into 2 ml vials and were kept at 4°C pending for analysis of VFA. Another 0.5 ml of supernatant was used for detection of non-VFA (lactic and succinic acids) using fumaric acid as internal standards. The concentrations of VFA and non-VFA were determined by gas chromatography (Agilent Technologies, USA) with a flame ionization detector (FID) and fused silica capillary column (30 m × 25 µm, inside diameter).

3. RESULTS

In the present study, mulberry silage was used to isolate some LAB as potential probiotic strains. A total of 50 individual colonies that showed typical characteristics of LAB colonies, were picked from MRS agar plates, subcultured and purified. After initial identification by catalase test and gram staining, only 38 isolates that were catalase negative and gram positive were selected for acid tolerance test. Of those 38 isolates, 34 strains were able to grow in MRS broth after 3 h exposure to pH 3, and of those, 23 isolates showing better acid tolerance [at least 90% growth, in comparison with that of the control (100%)] than the others were chosen for bile tolerance study. Of the 23 isolates tested for bile tolerance, 21 isolates were able to grow in presence of 0.3% bile salt, and of those, only 10 isolates with higher bile tolerance [at least 50% growth, in comparison with that of the control (100%)] than the others were selected and identified using 16S rRNA gene sequences.

Results of acid and bile tolerance tests of the 10 selected LAB are shown in Figure 1 and 2, respectively. The results showed that all 10 selected LAB had good acid and bile tolerance. In the acid tolerance study, the growth of all 10 strains after 3 h exposure to acidic condition (pH 3) was similar to their growth in normal condition. Furthermore, results of acid tolerance test showed that out of 10 isolates, three isolates (ITA19, ITA 23 and ITA 44) showed better growth after exposure to acidic condition than the control condition. Although, in bile tolerance study, none of the isolates showed better growth in presence of 0.3 % oxgall than the control condition, all the 10 isolates could grow (at least 50% growth in comparison with the control) in presence of 0.3% oxgall.

he results of identification of isolates using 16 S rRNA gene sequences are shown in Table 1. All the 10 isolated strains belonged to the genus *Lactobacillus* including one isolate similar to *L. pentosus*, two isolates similar to *L. farraginis*, two isolates similar to *L. brevis* and five isolates similar to *L. acidipiscis*. Since the results of identification revealed that some of the isolated strains were from the same species, we only chose one strain from each species (total of four strains) for further studies on organic acid production profile. The 16S rRNA gene sequences of the four selected *Lactobacillus* strains were deposited in the GenBank database under the accession numbers of KF297813 to KF297816 for isolates ITA22, ITA23, ITA33 and ITA44, respectively (Table 1).

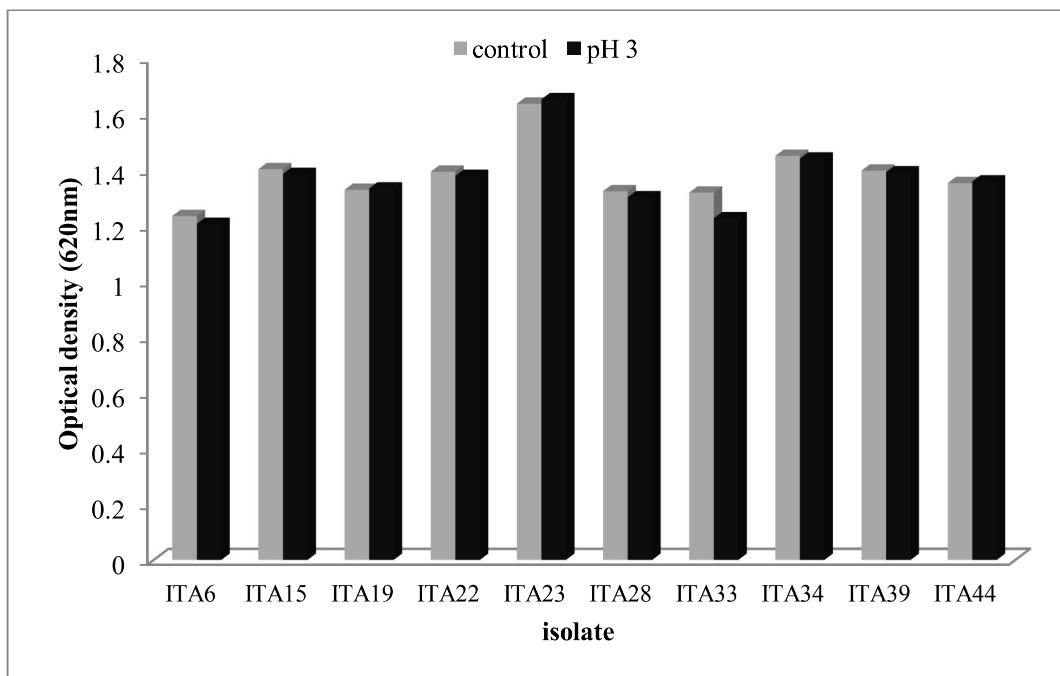

Figure 1. Results of acid tolerance test of 10 selected lactic acid bacterial isolates.

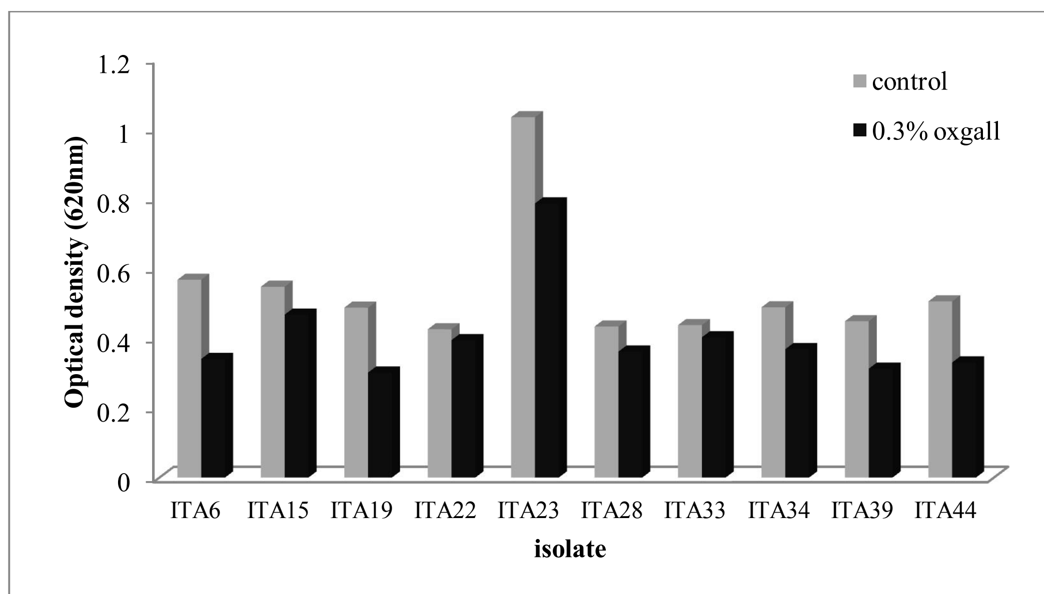

Figure 2. Results of bile tolerance test of 10 selected lactic acid bacterial isolates.

Table 1. NCBI blast results of selected isolates

Isolate	Accession No.	Nearest matched species from GenBank	Maximum score	Query coverage (%)	Maximum identity (%)
ITA22[1] (1398bp)	KF297813	*Lactobacillus farraginis*	2582	100	100
ITA23 (1390bp)	KF297814	*Lactobacillus pentosus*	2567	100	100
ITA33[2] (1401bp)	KF297815	*Lactobacillus brevis*	2588	100	100
ITA44[3] (1404bp)	KF297816	*Lactobacillus acidipiscis*	2562	100	99

[1]Same as ITA15; [2]Same as ITA38; [3]Same as ITA6, ITA19, ITA34 and ITA39

A phylogenetic tree based on 16S rRNA gene sequence analysis is shown in Figure 3. The tree shows the phylogenetic relationships among the 4 *Lactobacillus* strains isolated in the present study and 18 *Lactobacillus* type strains obtained from the GenBank. *Escherichia coli* was used as outgroup. Strains ITA22, ITA23, ITA33 and ITA44 were monophyletic with *L. farraginis* (AB262732.1) (bootstrap value of 100%), *L. pentosus* (HM067026.1) (bootstrap value of 100%), *L. brevis* (HM130535.1) (bootstrap value of 100%) and *L. acidipiscis* (AB326356.1)(bootstrap value of 97%), respectively.

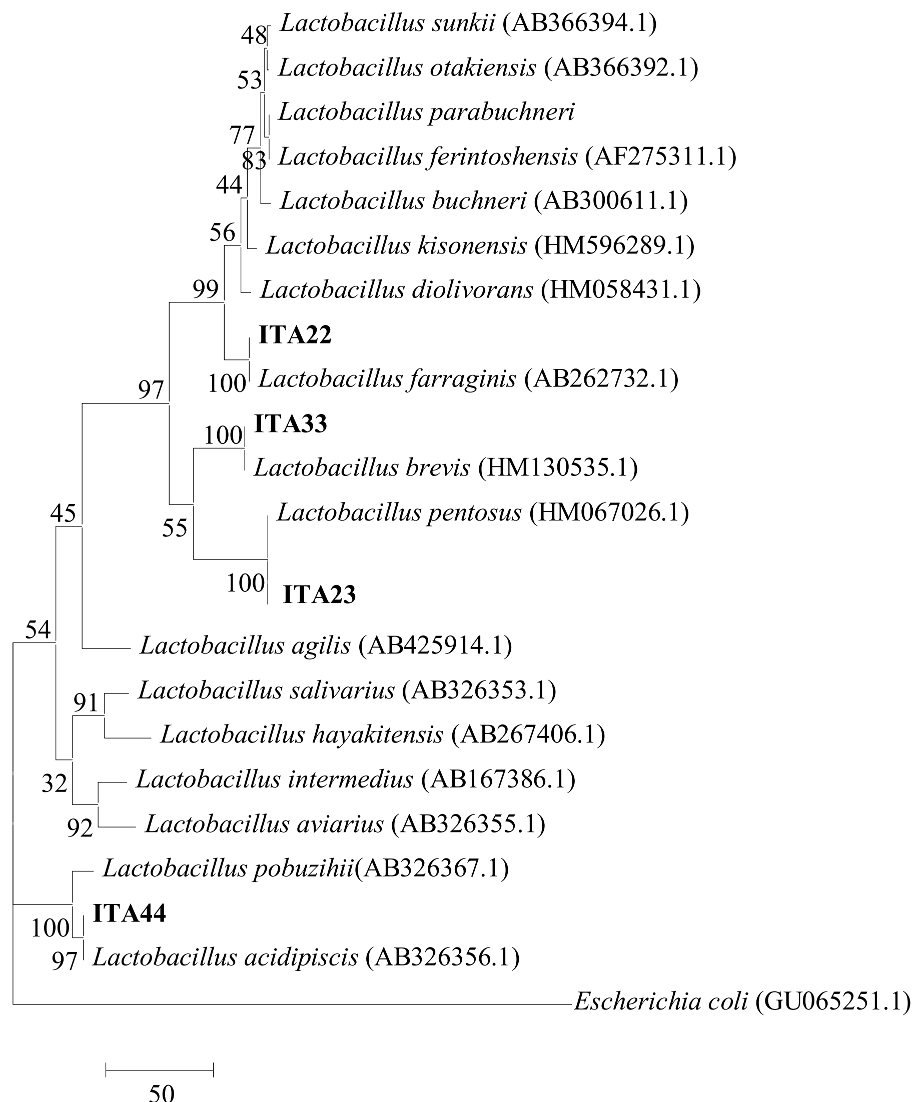

Figure 3. phylogenetic tree conducted based on the 16S rRNA gene sequences analysis. The evolutionary history was inferred using the Maximum Parsimony method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The analysis involved 23 nucleotide sequences. *Escherichia coli* has been used as outgroup. The scale is the branch lengths calculated using the average pathway method

Growth kinetics and reduction of pH of the growth medium

Results of growth rate of four selected *Lactobacillus* strains at 2, 4, 8, 12 and 24 h of incubation are shown in Figure 4. Among the four isolated *Lactobacillus* strains, *L. pentosus* ITA23 exhibited a more rapid growth from 2 to 8 h of incubation which corresponded to its exponential growth phase. However, in its stationary growth phase it had a more gradual growth from 8 to 24 h of

incubation. The growth rates of *L. farraginis* ITA22 and *L.brevis* ITA33 were almost similar with an approximately constant rate of growth from 2 to 24 h of incubation. *Lactobacillus acidipiscis* ITA44, however, showed a more rapid growth in its exponential growth phase from 4 to 12 h of incubation, while it had very little growth in its stationary growth phase from 12 to 24 h of incubation.

Figure 5 shows the reduction of pH levels of growth medium (MRS broth) by four selected *Lactobacillus* strains at 2, 4, 8, 12 and 24 h of incubation. *Lactobacillus farraginis* ITA22 and *L. pentosus* ITA23, reduced the pH of their growth media gradually from 4 to 12 h of incubation, however, the reduction of pH by these two strains was more rapid from 12 to 24 h of incubation. The pH of their growth medium after 24 h of incubation was 3.8 and 3.2, respectively. However, for the other two isolated *Lactobacillus* strains (*L.brevis* ITA33 and *L.acidipiscis* ITA44) reduction in the pH of growth medium was almost constant during the whole incubation period. The pH of their growth medium after 24 h of incubation was 4.1 and 3.7, respectively.

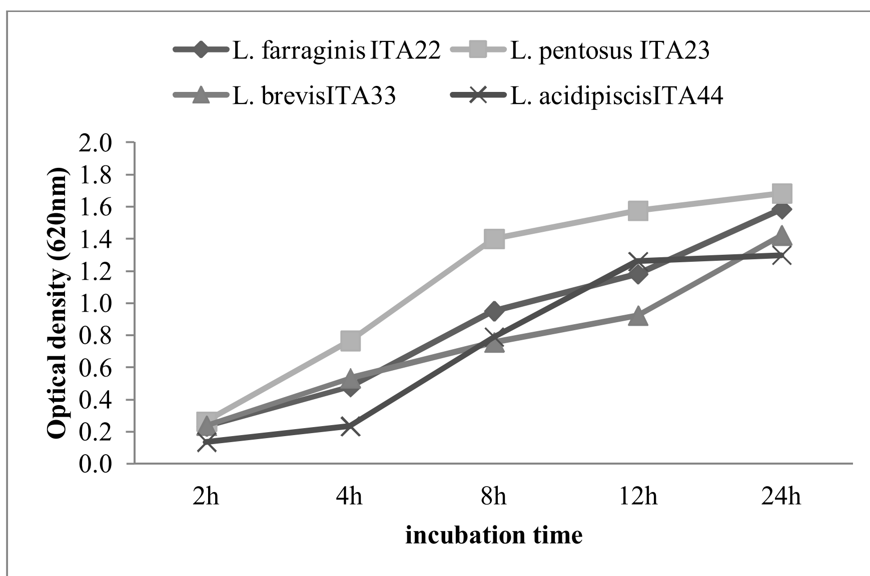

Figure 4. Growth kinetics of selected *Lactobacillus* strains during 24 h of incubation.

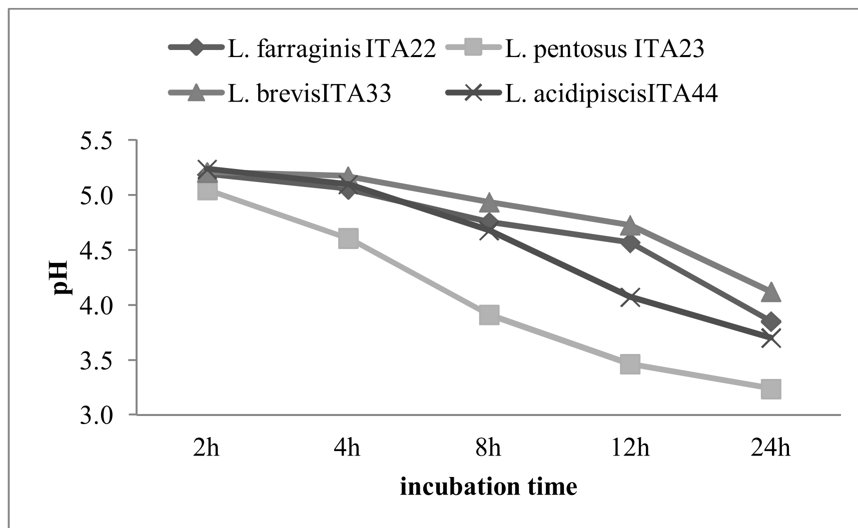

Figure 5. Reduction in pH of culture medium (MRS broth) by selected *Lactobacillus* strains during 24 h of incubation.

Organic acid production profile

The profiles of organic acid production of the four *Lactobacillus* strains are shown in Table 2. Lactic acid was the principal organic acid produced by the four *Lactobacillus* strains, followed by acetic acid. The amounts of lactic acid produced ranged from 433.41 (produced by *L. acidipiscis* ITA44) to 187.28 mM (produced by *L. brevis* ITA33) among the strains. However, 106.21 (produced by *L. farraginis* ITA22) and 86.79 mM (produced by *L. brevis* ITA33), were the highest and lowest amounts of acetic acid produced by the *Lactobacillus* strains, respectively. Succinic acid production varied widely among the *Lactobacillus* strains. *Lactobacillus farraginis* ITA22, produce high amount (39.27 mM) of succinic acid, while the other three *Lactobacillus* strains (*L. pentosus* ITA23, *L. brevis* ITA33 and *L. acidipiscis* ITA44) produced much lesser amounts of succinic acid (4.68, 3.17 and 6.55, respectively). Other acids such as propionic, isobutyric, butyric, valeric, isovaleric and caproic acids were either not produced or produced in trace amounts by the *Lactobacillus* strains.

Table 2. VFA and non-VFA production by LAB isolates after 24 h growth on MRS broth

	Non-VFA (mM)		VFA (mM)						
Lactobacillus strain	Lactic acid	Succinic acid	Acetic acid	Propionic acid	Isobutyric acid	Butyric acid	Isovaleric acid	Valeric acid	Caproic acid
L. farraginis ITA22	256.13	39.27	106.21	0.62	0.21	0.35	0.28	0.28	0.37
L. pentosus ITA23	533.97	4.68	91.14	0.97	0.16	0.14	0.2	ND	0.18
L. brevis ITA33	187.28	3.17	86.79	1.11	ND	ND	0.18	0.18	ND
L. acidipiscis ITA44	433.41	6.55	92.88	1.01	ND	ND	0.24	0.44	ND

ND, not detected

4. DISCUSSIONS

Lactic acid bacteria are the most commonly used bacteria as probiotic in food and feed industries (Metchnikoff and Metchnikoff, 1908; Collins *et al.*, 1998; Schrezenmeir and de Vrese, 2001) and different strains of LAB has already been isolated and characterizes as probiotic, but there is still keen interest to search for new strains which are more efficient than the existing ones. Efficiency and functional properties of every probiotic strain is importantly depended on the survival of the strain in the GIT of the host, in turn, is depended on the tolerance of the probiotic strain to the stressful environment of the host's GIT, where acid and bile salts are present. Therefore, tolerance to acid and bile is an important forefront requirement for selection of a potential probiotic strain (2007).

The results of the *in vitro* acid and bile tolerance tests very often predict the ability of the strains to survive in the host's body environment. However, acid and bile tolerance is only important for oral administration and may not be relevant for other applications of probiotics such as nutritional effect and antimicrobial ability (Ouwehand *et al.*, 1999). In the present study, assays of acid and bile tolerance of the isolated strains were carried out *in vitro*. The use of *in vitro* assays to initial assess of probiotic properties of new potential probiotic strains and select the most effective potential probiotic strains prior to *in vivo* investigations is necessary because the use of *in vivo* studies usually is time-consuming and expensive (Nemcova, 1997; Ehrmann *et al.*, 2002). In addition, it is suggested that adoption of proper criteria for the *in vitro* selection of probiotic bacteria can result in the isolation of strains capable of performing effectively in the GIT (2001).

In this study, pH 3 and 0.3% bile salt tests were used to assess the acid and bile tolerance of the isolated strains, respectively. That is because the pH in a human stomach containing food could reach to about 4 (Berrada *et al.*, 1991) and the normal concentration of bile encountered in human intestine is about 0.3% (Sjovall, 1959). Many studies also considered pH 3 (2005; 2008; 2011) and 0.3% bile salt (Gilliland *et al.*, 1984; Jacobsen *et al.*, 1999; Boonkumklao *et al.*, 2006; Koll *et al.*, 2008; Ruiz-

Moyano *et al.*, 2008; Sahadeva *et al.*, 2011) to determine acid and bile tolerance of probiotic strains, respectively.

The results of the present study showed that among the 38 tested isolates for acid tolerance, 34 isolates exhibited acid tolerance at pH 3 for 3 h, with 23 of those considered as good tolerated strains with at least 90% growth, in comparison with that of the control (100%). Ehrmann *et al.* (2002) reported that strains of *L. reuteri*, *L. salivarius* and *L. animalis* were able to tolerate pH 3 for 4 h. In a review by Charteris *et al.* (1998) it is mentioned that most of the *Lactobacillus* species are able to tolerate pH 4 for 1 h. Koll *et al.* (2008) suggested that bacterial strains which could tolerate pH 3 would be a good potential probiotic candidates for oral usage.

Among 23 tested isolates for bile tolerance in the present study, 21 strains were able to tolerate 0.3% oxgall. Similarly, Jacobsen *et al.* (1999), applying the same test procedure, reported that 41 of 42 tested LAB could tolerate bile at this concentration, while Koll *et al.* (Koll *et al.*, 2008) reported all the 67 LAB tested showed tolerance to this level of bile. Jin *et al.* (1998) also tested 12 strains of LAB for their tolerance to the same percentage of bile salt and found that all the tested strains were able to tolerate 0.3% of bile salt.

Since probiotic properties are strain specific (Dash, 1980) and extrapolation of the characteristics of a certain strain to another strain, even if it belongs to the same species, is not acceptable (1998), only well-identified and -characterized strains should be used as probiotic. FAO/WHO (2007) suggested that, every potential probiotic strain most be correctly identified by the most current and valid methodology for identification of probiotic strains. Hence, in the present study, after initial identification using catalase test, Gram staining and colony and cell morphologies, 16S rRNA gene sequence analysis has been used for identification of the isolated strains. 16S rRNA gene sequence analysis is an accurate, and reliable genotypic method for bacterial identification, which defines taxonomical relationships among bacterial strains (Petti *et al.*, 2005).

In the present study, among 21 isolates showing bile tolerance, 10 strains exhibiting higher bile tolerance than the others [at least 50% growth, in comparison with that of the control] were selected for identification to generic and species level using 16S rRNA gene sequences. The results of the molecular technique revealed that, as expected, the 10 selected strains were LAB, and belonged to the genus *Lactobacillus*. Based on the results of molecular identification some of the 10 isolated strains were from the same species, so only one strain from each species, namely *L. farraginis* ITA22, *L. pentosus* ITA23, *L. brevis* ITA33 and *L. acidipiscis* ITA44, were considered for further studies on organic acid production profiles.

Antimicrobial activity of LAB is well documented (Fernandez *et al.*, 2003; Coconnier-Polter *et al.*, 2005; Lonkar *et al.*, 2005; Liasi *et al.*, 2009; Mezaini *et al.*, 2009; Gaudana *et al.*, 2010; Sirilun *et al.*, 2010; Majidzadeh Heravi *et al.*, 2011) and it is attributed mostly to the production of antimicrobial substances such as organic acids, hydrogen peroxide and bacteriocins (Suskovic *et al.*, 2010). Among these substances, the most important ones are the organic acids. By production of organic acids and subsequently reduction of the pH level in the GIT, LAB are known to be able to reduce the population of pathogens in the intestine (Aroutcheva *et al.*, 2001). The toxic effects of organic acids produced by LAB are attributed to the reduction of intracellular pH and dissipation of the membrane potential (Kashket, 1987). In the present study, the four selected *Lactobacillus* strains reduced pH levels of their growth medium to the levels of 3.2 to 4.1 during the 24 h of incubation, which indicates on production of organic acids by the strains. Similarly, Boskey *et al.* (Boskey *et al.*, 1999) reported that eight vaginal *Lactobacillus* strains lowered the pH of their growth medium to pH of 3.2–4.8.

Organic acid production profiles of the four *Lactobacillus* strains showed that lactic and acetic acids, respectively, were the first and second abundant acids produced by all the strains. Since homofermentative *Lactobacillus* strains are known to ferment carbohydrates into energy and lactic acid (Jay, 2000; Reddy *et al.*, 2008), and heterofermentative *Lactobacillus* strains produces only 50% lactic acid and considerable amounts of acetic acid (Reddy *et al.*, 2008), it was expected that these two acids were produced in higher amounts by the *Lactobacillus* strains than the other acids. Because of production of organic acids by the four *Lactobacillus* strains isolated in the present study, it is expected that they could inhibit the growth of pathogenic strains. However, further study on the antagonistic effects of the strains should be conducted using different pathogenic strains to verify the antimicrobial activity of the strains.

The results of this *in vitro* study, indicated that among 38 isolated LAB from mulberry silage, the four selected *Lactobacillus* strains are able to survive in the GIT, and produce considerable amounts of organic acids, which make them to be considered as potential probiotic candidates for humans and animals. However, further studies are needed to characterize their probiotic properties such as antimicrobial activity, bacteriocin production, bile salt hydrolysis, etc.

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