Investigation of acid and bile tolerance of native lactobacilli isolated from fecal samples and commercial probiotics by growth and survival studies

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Abstract
This study aimed at applying both growth and survival approaches to compare three native strains of lactobacilli, belonging to *Lactobacillus plantarum, Lactobacillus rhamnosus* and *Lactobacillus acidophilus* species, with two commercial probiotic strains in their tolerance to acid and bile. The association between the data obtained from the methods was studied. The results of the different methods applied in this study, did not confirm each other for *all* the examined strains. However, the native strain of *L. plantarum* and the commercial strain of *L. acidophilus* repeatedly demonstrated the most and least bile resistances, respectively. The former excelled in all growth approaches but showed moderate acid resistance in the survival studies. Bile stress seemed to have more detrimental effects on all examined strains. The overall results suggest that the growth-rate designed studies and survival studies evaluating transit tolerance, might bring up different results when the examined strains belong to different species of lactobacilli showing different growth and metabolic activities. The strain of *L. plantarum* examined here could thus be considered as a potential probiotic, regarding its overall resistance to acid and bile.
Keyword: *Acid and bile tolerance; Probiotic; Lactobacilli; Growth; Survival*

INTRODUCTION

Today some strains of lactobacilli are extensively used in the food and pharmaceutical industries as commercial probiotics. Probiotics refer to viable microorganisms that promote or support a beneficial balance of the microbial population of the gut (Holzapfel and Schillinnger, 2002). It is most likely that the influence of probiotics is depending at least partly on the indigenous lactobacilli that are present in the gastrointestinal (GI) tract of the host (Waard *et al*., 2002).The diversity of these lactoflora varies between individuals depending on their genetic background, physiological and environmental factors (Arici *et al*., 2004). In addition, because of the strain dependency of health promoting properties of probiotics (Waard *et al*., 2004), it is necessary to find new probiotics among native strains. (Delgado *et al*., 2007; Khalil *et al*., 2007; Xanthopoulos *et al*., 1999).

According to the guidelines of the evaluation of probiotic organisms, reported by a joint FAO/WHO working group, two of the currently most widely used *in vitro* tests are resistance to gastric acidity and bile compounds based on both survival and growth studies (Vizoso Pinto *et al*., 2006). During the evaluation of bile tolerance by growth studies, the growth abilities of the examined strains in their culture media, containing different concentration of bile components can be assessed. These evaluations are obtained through the application of either of the following methods; a) by assessing the strain's ability in bringing about changes in optical density (Liong and Shah, 2005; Suskovic *et al*., 2000; Gilliand and Walker, 1990; Gilliland *et al*., 1984) and pH characteristics (Succi *et al*., 2005) of the broth culture media; b) by assessing growth ability on solid culture media (Morelli, 2007; Chou and Weimer, 1999; Prasad *et al*., 1999). The former, differentiates

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the bile resistant strains, growth rates of which are less affected by the inhibitory effects of the bile. A few growth studies have also addressed the evaluation of the acid tolerance of the examined strains (Nguyen *et al*., 2006). Instead, in survival studies, cell enumeration of the tested strains are carried out before and after keeping cells under stressful conditions resembling the stressful environment of the stomach or the small intestine transit. Such conditions could be introduced either separately or sequentially in which the latter could display possible interaction between theses stresses (Martin *et al*., 2006; Vizoso pinto *et al*., 2006; Succi *et al*., 2005; Alender *et al*., 1999; Charteris *et al*., 1998; Mustapha *et al*., 1997). Growth studies seem to be easier, more reproducible and cost effective than survival studies and the normal practice in screening for probiotic potentials usually begins with such examinations. Although all the above mentioned *in vitro* tests have been extensively used as the primary steps to select probiotic potentials, but rarely their correspondence in relation to one another has been disputed in the literature.

The objective of the present research was to apply growth and survival studies to evaluate the potential probiotic properties of three native strains of lactobacilli, isolated from infant's fecal flora with respect to acid and bile tolerance. There were two aspects in focus here (1) Comparison of native strains with commercial probiotic strains; (2) Compatibility of the growth and survival methods in discriminating amongst acid and bile resistant strains.

To date, this is the first trial involving comparison of acid and bile tolerance of commercial lactobacilli with *Lactobacillus* isolates originating from Iranian infants' fecal flora.

MATERIALS AND METHODS

Bacterial strains and chemicals: Three native *Lactobacillus* stains used in this study were selected from 33 isolates of lactobacilli originating from the fecal samples of 6 infants less than 2 years of age. They were isolated on de Man Rogosa Sharpe (MRS) (Sharlou-Spain) medium containing 1 mg/l Vancomycin, adjusted to pH 5.4 by lactic acid (Mirlohi *et al*., 2008a) and identified as *Lactobacillus acidophilus* H26, *Lactobacillus rhamnosus* L51 and *Lactobacillus plantarum* A7 based on biochemical tests (Mirlohi *et al*., 2008b). Identification of *Lactobacillus plantarum* A7 was also confirmed by specific Polymerase Chain Reaction (PCR), using a *reqA* gene derived primer, plantF (5′-CGTTTATGCGGAACACCTA-3′) and a general primer pREV (Torriani *et al*., 2001). Electrophoretic observation of the amplification products with the expected length of 318#bp showed that the given strain was a *L. plantarum* strain (unpublished data). *L. rhamnosus* GG was isolated from a pharmaceutical product (Culturelle, USA) and identified based on biochemical and molecular methods (Mirlohi, *et al*., 2008b). *L. acidophilus* was purchased from a dairy company (Lactina, Bulgaria) as a commercial probiotic dairy culture and its identity was confirmed based on biochemical and physiological criteria. As the strain of the given commercial *L. acidophilus* was unknown, in this study, it was arbitrarily named as *L. acidophilus* Lac. The cultures were stored at -80ºC in 15% (v/v) glycerol. During the experiments, stock cultures were maintained at 4ºC on MRS agar slants, and were subcultured monthly. Working cultures were activated by two successive transfers in sterile MRS broth using 1% (v/v) inoculums at 37ºC before each experiment. Oxgall (Sigma Chemical Co., St. Louis, MO, USA, cat. no. B-3883) was used as bile component; 8N hydrochloric acid and sterilized 8% (w/v) saturated sodium bicarbonate solution were used for adjusting pH to the desired level during the experiments.

Evaluation of bile and acid tolerance of the strains through growth studies: The bile resistance of the isolates was evaluated by the method of Gilliland *et al*., (1984). The *Lactobacillus* strains were grown overnight in MRS broth. One hundred µl of the culture suspension was then inoculated into the tubes containing 20 ml of MRS broth with or without 0.3% (w/v) Oxgall, the latter was considered as control. The inoculated tubes were incubated at 37ºC anaerobically, under 5% CO_2 in a CO_2-air-jacketed incubator (Memmert, Germany). For each strain, three independent tests, each in duplicate, were carried out. Growth was monitored every 15 min for 10 h by measuring optical density at 620 nm (OD_{620}) using a spectrophotometer (2100, UV-Vis, Unico, USA). The bile tolerance of each strain was defined as the difference in the time required for the absorbance value to increase by 0.3 units between MRS containing Oxgall and the control (Liong and Shah, 2005; Patal *et al*., 2004; Prasad *et al*., 1998; Gilliland and Walker, 1990). Bile tolerance was also evaluated through another growth study (Succi *et al*., 2005). In this procedure, the ability of the examined strains to reduce the pH in the presence of

different percentages of bile salts or Oxgall was considered as the bile tolerance. One percent (v/v) of inocula of the activated culture medium of each strain were transferred to MRS broth containing 0, 0.3, 0.5, 1, 1.5 and 2% (w/v) bile component and the changes in pH were monitored (pH/temperature tester, Eutech instrument, Malaysia) at time intervals of 3, 6, 24 and 48 h.

In the evaluation of acid tolerance of the tested strains by the growth study, the method used by (Negugen, *et al*., 2006) was applied. One percent inocula (w/w) of the activated cultures in MRS broth, acidified to pH 3 with 8N hydrochloric acid were prepared in duplicates. The strains capable of growing to $>10^7$ CFU/ml after 24 h (37°C, 5% CO_2) were considered as acid resistant strains. Each of the last two experiments is characterized by two independent duplicate tests.

Evaluation of acid and bile tolerance of the strains through survival studies: Survival study was performed based on the method used by (Succi *et al*., 2005). Activated cultures of each strain were inoculated at 1% (v/v) concentration into 100 ml of MRS broth acidified to pH 2.5 or 3 with 8N hydrochloric acid. The OD_{620} of all broth cultures was adjusted to 1.5, with a maximum difference of 0.25 between the broth cultures. They were incubated at 37ºC under 5% CO_2 for 1 and 2 h. This was followed by increasing the pH of the culture medium with saturated sodium bicarbonate solution to 6.8-7.0 and subsequent addition of 1% (w/v) Oxgall. The incubation was continued for more than 4 h under the same conditions mentioned above. The survival of the tested strain was assessed by sampling of the medium at time intervals of 0, 1, 2 and 4 h during the 6 h of the experiment. Ten fold serial dilutions were made from each 1ml sample using peptone water and then, pour plated on the MRS agar. Approximately 30-250 colonies appeared after 48 h of incubation at 37ºC under a 5% CO_2 concentration. The experiment was repeated twice for each strain and the data was presented as mean.

Statistical Analysis: Data analysis was carried out with the Minitab software (version 15). One way analysis of variance (ANOVA) was used to determine significant difference between the means, with significant level at $\alpha = 0.05$.Tukey's test was used to perform multiple comparisons between the means. In all growth studies, the mean of two to three times repeated measurements yielded the value for each replicate.

RESULTS

Growth studies: Table 1 presents the results of the mean values of the time required for each strain to increase by 0.3 units. As seen in Table 1, both *L. plantarum* A7 and *L. rhamnosus* GG showed superior growth rates when compared to the other strains tested, but after the addition of 0.3% (w/v) Oxgall, only *L. plantarum* A7 displayed the best growth ability. *L. acidophilus* H26 and *L. rhamnosus* L5K1 exhibited nearly the same bile tolerance as that of *L. plantarum* A7.

The results of the analysis of the mean pH values of the inoculated MRS and MRS supplemented with 0.3 and 0.5% (w/v) Oxgall, are represented in Table 2. In MRS without bile, *L. plantarum* A7 grew faster than the other strains with growth appearing after 6 h of inoculation. This strain together with *L. acidophilus* Lac and *L. rhamnosus* GG exhibited higher abilities in decreasing the pH of the MRS medium after 24 h of inoculation in comparison with *L. rhamnosus* L51 and *L. acidophilus* H26. By incorporation of 0.3% (w/v) and 0.5% (w/v) Oxgall in the MRS medium, *L. plantarum* A7 and *L. rhamnosus* GG appeared to be more

Table 1. Bile tolerance of *Lactobacillus* strains based on the time required for their optical density to increase 0.3 units[1].

Time / Strain	H1	H2	H2-H1
L. plantarum A7	2.81 ± 0.17^{a}	3.70 ± 0.26^{a}	$1.08^{a} \pm 0.06$
L. rhamnosus L51	4.75 ± 0.19^{b}	6.92 ± 0.34^{b}	2.23 ± 0.48^{ac}
L. acidophilus H26	4.88 ± 0.36^{b}	5.65 ± 0.73^{b}	1.41 ± 0.32^{ac}
L. rhamnosus GG	3.622 ± 0.17^{a}	7.03 ± 0.84^{b}	3.41 ± 0.40^{bc}
L. acidophilus Lac	4.67 ± 0.14^{b}	>9	*

[abcd]Means within a column without a common superscript are significantly different ($p< 0.05$) according to Tukey's test. [1]Triplicate trials, producing four observations. Results are expressed as means ± standard deviation of means. *, H1: Time (h) required for OD_{620} to increase by 0.3 units in MRS, H2: in MRS+0.3% oxgall. *undetermined.

Table 2. Bile tolerance of *Lactobacillus* strains based on the pH changes of the cultured media[1].

Cultured media	MRS					MRS+0. 3%0xgall					MRS+0.5%0xgall				
Sampling time (h)	0	3	6	24	48	0	3	6	24	48	0	3	6	24	48
Strain															
L. plantarum A7	5.80± 0.03^{a}	5.61± 0.08^{a}	5.10± 0.02^{a}	3.73± 0.06^{a}	3.61± 0.07^{a}	5.85± 0.01^{a}	5.77± 0.05^{a}	5.43± 0.27^{a}	3.72± 0.04^{a}	3.69± 0.11^{a}	5.89± 0.05^{a}	5.73± 0.06^{a}	5.50± 0.10^{a}	3.92± 0.02^{a}	3.86± 0.05^{a}
L. rhamnosus 5K1	5.81± 0.07^{a}	5.70± 0.06^{ac}	5.50± 0.09^{b}	4.60± 0.24^{b}	3.80± 0.02^{b}	5.92± 0.03^{a}	5.88± 0.02^{a}	5.87± 0.02^{b}	5.80± 0.03^{b}	4.77± 0.06^{b}	5.89± 0.02^{a}	5.88± 0.04^{b}	5.89± 0.46^{b}	4.93± 0.08^{b}	4.92± 0.06^{b}
L. acidophilus H26	5.87± 0.03^{a}	5.89± 0.02^{bc}	5.62± 0.07^{b}	4.29± 0.10^{b}	3.94± 0.02^{bc}	5.96± 0.03^{a}	5.91± 0.02^{a}	5.93± 0.06^{b}	5.55± 0.08^{b}	4.76± 0.03^{b}	6.02± 0.05^{a}	5.92± 0.07^{b}	5.92± 0.07^{b}	4.85± 0.16^{b}	4.88± 0.09^{b}
L. rhamnosus GG	5.81± 0.06^{a}	5.79± 0.06^{ac}	5.54± 0.09^{b}	3.83± 0.20^{ab}	3.54± 0.09^{a}	5.87± 0.05^{a}	5.73± 0.01^{a}	5.4± 0.02^{bc}	3.90± 0.02^{a}	3.69± 0.11^{a}	5.95± 0.01^{a}	5.78± 0.00^{b}	5.24± 0.32^{a}	4.44± 0.09^{a}	4.08± 0.04^{a}
L. acidophilus Lac	5.82± 0.07^{a}	5.76± 0.03^{ac}	5.44± 0.14^{b}	3.71± 0.13^{ac}	3.67± 0.08^{ac}	5.74± 0.15^{a}	5.79± 0.09^{a}	5.89± 0.01^{b}	5.56± 0.01^{b}	4.76± 0.02^{b}	5.95± 0.06^{a}	5.95± 0.02^{b}	5.95± 0.04^{b}	5.96± 0.02^{c}	5.92± 0.05^{c}

[1]Duplicate trial, producing four observations. Results are expressed as the means of pH values ± standard error of the means.
[abcd]Means within a column without a common superscript are significantly different ($p<0.05$) by Tukey's test.

Table3. Acid tolerance of *Lactobacillus* strains based on the growth study[1].

strain	Log_{10} CFU/ml*	Log_{10} CFU/ml**
L. plantarum A7	7.11 ± 0.51	8.39 ± 0.17
L. rhamnosus L5k1	7.01 ± 0.09	7.67 ± 0.04
L. acidophilus H16	7.85 ± 0.02	7.42 ± 0.13
L. rhamnosus GG	7.09 ± 0.33	8.02 ±0.23
L. acidophilus Lac	6.68 ± 0.18	7.23 ±0.63

[1]Results represent means of two independent duplicate experiments ± standard deviation of means.
*Bacterial count immediately after inoculation. **Bacterial count after 24 h.

tolerant than other strains. Instead, *L. acidophilus* Lac was shown to be the most sensitive strain under the same conditions. In the presence of 1-1.5% Oxgall (w/v), *L. plantarum* A7 and *L. rhamnosus* GG showed signs of growth after 48 h, but the other three strains were highly inhibited. None of the strains grew in the presence of 2% (w/v) Oxgall (data are not shown).

The results of the mean values of the viable cells present in the inoculated MRS (pH 3) immediately after inoculation and also after 24 h are represented in Table 3. All the tested organisms were able to grow in acidic media, however, *L. plantarum* A7 and *L. rhamnosus* GG grew better than the other strains resulting in more than 10^8 CFU/ml after 24 h of incubation.

Survival studies: The results of the effect of acid and bile stress on the survival of the examined strains are shown in Figure 1. Black and white arrows indicate the first exposure to acidic MRS and MRS containing bile, respectively. Both commercial strains, *L. acidophilus* Lac and *L. rhamnosus* GG have been shown to be more resistant to acid stress (pH 3) than the three native ones and exposure to the harsher acidic media (pH 2.5) made this difference even more significant. Damage to the commercial strains (*L. acidophilus* Lac and *L. rhamnosus* GG) due to the acidic environment was revealed by decreases of 0.3-0.7 and 0.35 - 0.97 log_{10} CFU/ml, respectively after 2 h of incubation in acidified MRS (pH 2.5).Whereas, decreases of about 0.46-2.54, 0.45-2.86 and 1.9-2.2 log_{10} CFU/ml were observed for the 3 native strains of *L. plantarum* A7, *L. rhamnosus* L5k1 and *L. acidophilus* H26, respectively. After being in acidic medium (pH 3), subsequent exposure to the neutralized bile containing environment seemed to have a more adverse effect on the survival of the examined strains with the exception of *L.*

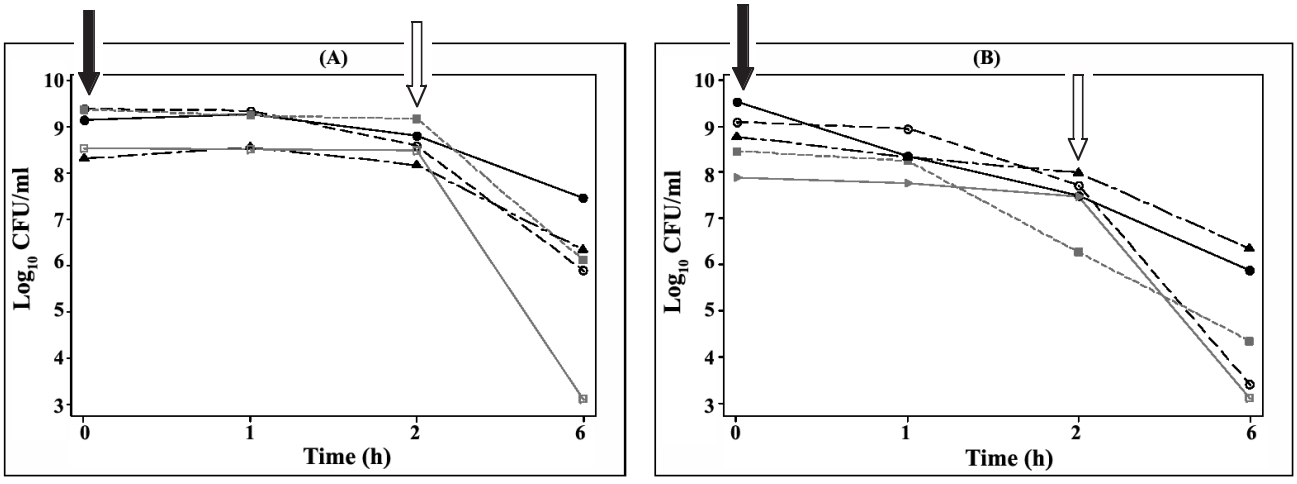

Figure1. Effect of acid (A: pH 3, B: pH 2.5) and bile stress on viability (log CFU/ml) of *L. plantarum* A7 (●), *L. rhamnosus* GG (▲), *L. rhamnosus* L5k1 (O), *L. acidophilus* H16 (■) and *L. acidophilus* Lac (□).

plantarum A7 and *L. acidophilus* H26. They showed further decreases of 1.33-1.6 and 0.8-1.69 $\log_{10}$ CFU/ml under this condition. *L. rhamnosus* GG and *L. rhamnosus* L5k1 were more sensitive and their cell counts was decreased by 1.51-2.153 and 2.27-3.49 $\log_{10}$ CFU/ml, respectively (Fig. 1A). Using a stronger acidic medium (pH 2.5), subsequent exposure to bile, noticeably decreased the cell number of *L. rhamnosus* L5k1 to less than 4 $\log_{10}$ CFU/ml. While, *L. rhamnosus* GG and *L. plantarum* A7 did not show further reductions (Fig. 1B) which compared to what was observed before (Figure 1A). Despite the strong acid tolerance characteristic, *L. acidophilus* Lac revealed remarkable sensitivity to bile stress, resulting in a lower microbial count of 5-6 $\log_{10}$ CFU/ml. This reduction made it the least survivable microorganism among the tested strains.

DISCUSSION

The results of the growth studies, which obtained from the two tested methods, did not support each other in evaluating the bile tolerance of *L. rhamnosus* GG, *L. rhamnosus* L5K1 and *L. acidophilus* H26. It could be concluded that a major difference in bile resistance of the strains is needed to yield the same results using the two mentioned methods.

Chateau *et al*. (1994) suggested that lactobacilli could be classified into four groups according to their delay in growth in the presence of bile (d): resistant strains ($d \leq 15$ min), tolerant stains ($15 \text{ min} < d \leq 40$ min), weakly tolerant stains ($40 \text{ min} < d < 60$ min) and sensitive strains ($d > 60$ min). According to this classification, all the strains used in this study are bile sensitive. As the commercial probiotic microorganisms used in this study, were also categorized as the sensitive strains, it is most likely that this classification is not valid. In addition, the results of this research are different from those of Succi *et al*. (2005). In the present study, bile stress led to greater inhibitory effects when compared to those observed by Succi and colleagues. Also, there is a particular difference between the results of the present study and the result of Suskovic *et al*. (2000) regarding the performance of growth studies in evaluation of the bile tolerance of *Lactobacillus* strains. The latter study claimed that 8 h following inoculation, the treated and control cells of a *L. acidophilus* strain entered the stationary phase of growth and the final cell count remained constant. In contrast to this finding, despite the inhibitory effects of bile (Table 2), the examined strains in this study continued growing in bile containing medium, 8 h after inoculation.

Oxgall is a natural dried bovine bile component containing both conjugated and unconjugated bile salts. Conjugated bile salts comprise a combination or mixture of bile derivatives in which their ratios vary from one individual to another. Depending on the peptide residues and dissociation constant and also bile salt hydrolase activity, conjugated bile salts result in different toxicity effects when compared to one another (Begley *et al*., 2006; Patel *et al*., 2004). Therefore, the differences between the results of this study with those of others, could be attributed to the difference between the bile components used.

The results of the survival studies confirmed the observation of other studies in which the commercially used probiotics were less tolerant to either acid are bile or artificial gastric and duodenum juices, when compared to noncommercial strains (Vizoso pinto *et al*., 2006; Prasad *et al*., 1999). Long-term subculturing and several passages of the commercial strains might lead to the development of less tolerant genetic variants in industry. (Tuomola, 2001).

Some studies have shown that the presence of bile salts in the bacterial culture medium is much more detrimental than the effects of low pH (Khalil *et al*., 2007). This claim supports the results of the present study, in that the bile stress leads to more inhibitory effects than acid stress. However, the results of this study are different from those of other studies in which the main decrease in survival of the examined strains has been shown to occur in acidic medium (Succi *et al*., 2005).

Correlation between growth and survival studies: In the evaluation of the compatibility of the methods applied, the results of the growth studies carried out for assessing bile tolerance were in accordance with the survival studies of *L. plantarum* A7 and *L. acidophilus* Lac, which showed the best and the least characteristics in this property. In the evaluation of acid the results of growth studies were not in agreement with those of the tolerance, survival studies. It is most likely that the mechanisms involved in acid resistance in lactobacilli (Cotter and Hill, 2003) do not support the growth rate, to the same extent that they protect cells' survival under acidic stressful environments. For instance, among the mechanisms responsible for acid tolerance in *L. acidophilus* strains, cell wall integrity and biochemical structure are considered

as key factors in protecting the cells from acidic conditions (Frece *et al*., 2005; Conway *et al*., 1986). It is unlikely that these characteristics compensate the growth delay in an equal manner. Hence, it would be reasonable to observe different responses from a *Lactobacillus* strain in growth and survival studies as the result of this study revealed for the commercial *L. acidophilus* strain. Alternatively, despite the strain dependency of probiotic properties, when different species with noticeable differences in metabolic activity are challenged by a stressful factor in growth rate, the intensity of their responses to the stress factor might be different depending on their natural growth abilities. As an example, *L. plantarum* is known as the most adaptable *Lactobacillus* species due to its large genome, capability in metabolizing different carbon sources and growth ability. By having such characteristics, it enables such a species to colonize different environments (Morelli *et al*., 2004; Molin, 2001; Kandler and Weiss, 1986). Therefore, when growth rate is under investigation, the strains belonging to this species may demonstrate higher resistance than others. Hence, *L. plantarum* and *L. acidophilus* are very different in biochemical and physiological properties; however, a species-related bile resistance has also been observed amongst the closely related species (Morelli, 2007).

CONCLUSION

The results of this study, confirm the preposition that acid and bile tolerant strians do exist in the population (Cebeci and Gurakan, 2003). Accordingly, the strain *L. plantarum* A7 can be identified as a potential probiotic strain with respect to *in vitro* acid and bile experiments. As one of the commercial probiotics, examined here, was lack of bile resistancy, it is thus recommended that food industries and laboratories examine the imported commercial products regarding their probiotic claims before their purchasing. In this way, introduction and development of native strains with an identified origin and specific probiotic features can be very valuable. The results of this study further suggest that when the delay in growth rate is regarded as the point of comparison, the species specificity of the *Lactobacillus* strains could be considered as an effective parameter. As *in vitro* studies can only partially mimic the actual *in situ* conditions in the gut ecosystem, survival of strain A7 under conditions more similar to the human GI tract could provide more clear information regarding the characterization of native probiotic strain. This study is currently in process.

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