Screening of *Lactobacillus* isolates for their adherence capabilities to mammalian cells and their acid and bile tolerance

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Abstract

Background: Lactobacilli resemble a major part of the commensal human mucosal flora. The application of *Lactobacilli* as probiotics has increased during the last few years since a health promoting effect has been reported in addition to their long history of safe use.

Methods and finding: In this study, fifty-two *Lactobacillus* isolates were recovered from dairy products or infant stools were examined *in vitro* for their probiotic potential. Their adherence capacities to *Vero* cells in addition to their acid and bile tolerance were evaluated. Only few isolates weren't able to adhere to *Vero* cells, while the other isolates have high to moderate adherence capacities. The majority of isolates were tolerant to acid and about 70% were tolerant to 0.3% bile salts.

Conclusion: thirty two *Lactobacillus* isolates were found to possess desirable probiotic properties. These isolates are good candidates for further investigation in *in vitro* and *in vivo* studies for their potential health benefits and their application as novel Biotherapeutic agents.

Keywords: Probiotics – Lactobacilli – Adherence to mammalian cells – Acid and bile tolerance

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Introduction

Lactobacilli are Gram-positive, mostly facultative but under certain conditions strictly anaerobic, non-sporulating rods. These bacteria have a long history of safe use, especially in the dairy industry [1]. Lactobacilli resemble a major part of the commensal human mucosal flora [2-6].Clinical studies could demonstrate a protective role of lactobacilli for intestinal infections and urogenital as well as a capability to prevent and treat allergic diseases [7,8]. Increased drive has existed for the isolation of novel *Lactobacillus* strains that exert a beneficial health effect when ingested by humans. Such strains are termed probiotic. Probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host [9].

In order for a probiotic strain to exert its beneficial effect on

the host, it has to be able to survive passage through the host's digestive tract [10]. The ability of *Lactobacillus* strains to adhere to the mucosal surfaces of the intestine and the subsequent colonization has long been one of the most commonly encountered criteria for the selection of probiotic strains. Adhesive probiotic lactobacilli have been reported to have beneficial health effects, especially related to the inhibition of pathogen adhesion to intestinal cell lines.

The aim of this study was to evaluate the probiotic potential of *Lactobacillus* isolates recovered from dairy products as well as infant stool by applying *in vitro* screening tests and to select probiotic candidates that fulfill the established criteria and could therefore be potentially used as novel therapeutic agents.

Materials and Methods

Materials and their sources

Dolbeco modified Eagles Media (DMEM), Trypsin 0.25% - EDTA solution (sterile filtered), Fetal bovine serum (sterile filtered) was purchased from Sigma Aldrich Co.,USA. Rogosa agar (Oxide), deMan Rogosa Sharpe *(*MRS) broth (Difco), Bile Salts Mixture (Starchemic, India), Flat bottom sterile tissue culture plates of 96 wells (Nunclon, Denmark), BD BBL Gas-Pak™ 100 System, (Becton and Dickison company, USA),Gas Generating Kit (Oxoid).

Isolation and identification of *Lactobacillus*

Lactobacillus isolates were recovered from dairy products (raw milk, butter, cheese and yoghurt), stool specimens (from breast-fed infants) as well as freeze dried lactic culture (REDI-SET, used for Bulk starter culture, purchased from Chr. Hansen`s Lab., Denmark). Milk and processed milk products were obtained from farmers.

Isolation of Lactobacillus from dairy products

About 1 g of each sample (raw milk, cheese, yoghurt and butter) was taken aseptically, transferred to 10 ml phosphate buffered saline and then vortexed [11]. An aliquot (1 ml) of the homogenate produced was then inoculated in 10 ml MRS broth contained in screw caped tube, and incubated under anaerobic conditions (GasPak Anaerobic System) for 24 h at 37°C. An aliquot (50 µl) of the obtained culture was spread on acidified MRS agar plates, acidified with glacial acetic acid to pH 5.7 and incubated anaerobically for 48 h at 37°C. Colonies with typical characteristics of *Lactobacillus* (small, white, circular, entire margin with diameter 1-3 mm) were selected and subcultured in MRS broth for further purification and identification.

Isolation of Lactobacillus from infant stool

Lactobacilli were isolated from stools of healthy breast-fed infants (aged 3-6 months). One gram from a fresh fecal sample (maximum lag time was 2 h) was suspended in 9 ml of a 0.85% NaCl solution (pH 7.0). The slurry was homogenized and filtered to remove any large particles and debris [12]. For enrichment of lactobacilli, all the samples were diluted and cultured in deMan Rogosa Sharpe (MRS) broth at 37 °C for 48 h under anaerobic conditions, followed by plating on MRS or Rogosa agar. Isolated colonies with typical characteristics of lactobacilli were picked from the plates and further purified on MRS or Rogosa agar [13].

Recovery of Lactobacillus from Bulk starter culture

One gram of the dried lactic culture were suspended in 100 ml MRS broth, incubated at 37 °C for 48 h under anaerobic conditions, followed by subsequent subculture in MRS broth then, the obtained growth plated on acidified MRS agar plates (acidified with glacial acetic acid to pH 5.2). Isolated colonies with typical characteristics of lactobacilli were picked from the plates and further purified on MRS.

The collected isolates were stored at 20°C in MRS broth containing 20% glycerol [14].

Identification of the selected colonies.

The collected isolates were identified to genus level by microscopical examination and catalase and oxidase test [11,15-16].

Mammalian cell line

The cell line used in this study was *Vero* Cell Line *(*ATCC No. CCL-81*)*, are kidney epithelial cells derived from African green monkey and was purchased from VACSERA, Egypt.

Screening the *Lactobacillus* isolates for their adherence capabilities to mammalian cells

Lactobacillus isolates were cultured anaerobically in MRS broth at 37°C. The 24-hours MRS culture of tested *Lactobacillus* was centrifuged, washed twice with phosphate buffered saline pH 7 and then resuspended in DMEM and the count was standardized to 10^8 CFU/ml in the same medium. To evaluate binding of *Lactobacillus* isolates to epithelial cells, *Vero* cells which supplied as a confluent monolayer in 96-well tissue culture plates was washed and then, 200 µl aliquots of 10^8 CFU /ml *Lactobacillus in* DMEM were added to each well. After 2 hours of incubation at 37°C, cell culture medium was aspirated off and cells were washed three times with DMEM-PBS (1:1), (pH 7.4, 37°C) to remove non-bound bacteria. Cells were released from polystyrene wells by adding 0.125 ml aliquots of 0.05% trypsin-EDTA to each well and incubat-

ing the plate at 37°C for 30 minutes [17]. Serial dilutions of bacteria were plated on MRS agar and the agar plates were incubated anaerobically for 48 h at 37°C for subsequent CFU quantification.

Testing the survival of *Lactobacillus* isolates under conditions simulating the human GI tract

The tested *Lactobacillus* isolate was cultured anaerobically in MRS broth at 37°C. Bacterial cells from overnight (18 h) culture were harvested at 5000 rpm, 10 min, and then they were washed twice with PBS buffer, pH 7.2, before being suspended in the same buffer. The bacterial count was adjusted to 10^8 CFU/ml (optical density 0.2 at 600 nm).

Acid Tolerance

The resistance of the examined lactobacilli to low pH environment was tested as described by *Maragkoudakis* [18]. Aliquots of 100 µl of the prepared bacterial suspension were added to 10 ml quantities of MRS broth contained in screw caped tubes adjusted to pH 2 or 3 to mimic gastric acidity and pH 6.2 as control. Initial populations was about 10^6 CFU/ml. Resistance to acid was assessed in terms of viable colony counts and enumerated after incubation at 37°C anaerobically for 1 and 3 h, reflecting the time spent by food in the stomach. An aliquot of 100 µl was taken at 0, 1 and 3 h from each tube of specified pH, serially diluted and then plated on MRS agar and incubated anaerobically for 48 h at 37°C.

Bile Tolerance

Tolerance of *Lactobacilli* to bile was evaluated by examining the survival of the tested isolates in presence of 0.3% w/v bile salt mixture (Starchemic, India) which mimics the bile concentration of the human gastrointestinal tract. Aliquots of 100 µl quantity of the prepared bacterial suspension were added to 10 ml quantities of MRS broth contained in screw caped tubes, pH 6.2, enriched with 0.3% w/v bile salts and MRS broth having pH 6.2 as control. Initial populations was about 10^6 CFU/ml. Resistance to bile was assessed in terms of viable colony counts and enumerated after incubation at 37 °C anaerobically for 1 and 4 h, reflecting the time spent by food in the small intestine. An aliquot of 100 µl was taken at 0, 1 and 4 h from each tube, serially diluted and then plated on MRS agar and incubated anaerobically for 48 h at 37°C [18]. All screening experiments were done in triplicates, the presented data were average of three values.

Results

Isolation and identification of *Lactobacillus*

From the different samples collected from various sources (raw milk, cheese, butter, yoghurt as well as infant stool), 89 bacterial isolates and 12 yeast isolates were recovered.

The isolates that showed the *Lactobacillus* characteristics according to colony morphology on MRS agar (appeared as small, white, circular, entire margin with diameter 1-3 mm), Gram staining (Gram positive bacilli, non-endospore forming), catalase negative, oxidase negative were selected. These isolates (52) included; 35 isolates were recovered from raw milk, 8 isolates were recovered from cheese, 7 isolates were recovered from butter, 1 isolate (LS) from a freeze dried lactic culture and 1 isolate (S1) was recovered from infant stool.

Adherence of different *Lactobacillus* isolates to Vero cells

The adherence of the collected *Lactobacillus* isolates (52 isolates) to *Vero* cells was investigated and the adhered cells were expressed as percentage of initial count. The adherence capabilities to *Vero* cells varied greatly among the tested *Lactobacillus* isolates, ranging from 0.001% to 6.4% (**Figure 1**).

The number of bacteria adherent per *Vero* cell (adherence capacity) of the tested *Lactobacillus* isolates was shown in **Table 1.** The tested *Lactobacillus* isolates were categorized according to their relative adherence capacities. About 50% of the tested isolates *Lactobacillus* showed good adherence, about 40% showed moderate adherence while, 10% having no adherence capacity to *Vero* cells.

The selection of the *Lactobacillus* isolates for further testing was dependant on their adherence capacities to *Vero* cells. Isolates showed no adherence capacities to *Vero* cells were excluded, while the other isolates were further tested to investigate their probiotic potential.

Survival of the tested *Lactobacillus* isolates under conditions simulating GIT

The tolerance of *Lactobacillus* isolates, which showed adherence capacity > 0.1 adhered bacteria / *Vero* cell (46 isolates), to acid and bile was tested.

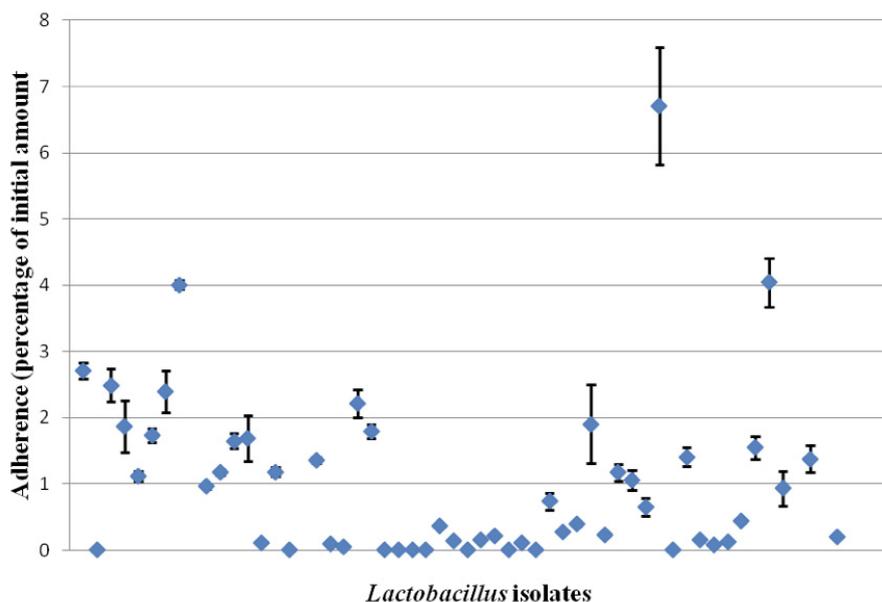**Fig.1.** Scatter plot of Adherence of fifty two Lactobacillus isolates to Vero cells

Table 1. Adherence capacity of the tested Lactobacillus isolates to Vero cells.

Adherence capacity range	Isolate Code*	Adherence capacity value**	number of isolates	percentage (relative to total number of isolates)
High adherence (more than 50 adhered bacteria / Vero cell)	L47	167±22	9	17%
	C1	84±3.7		
	C10	84±1.4		
	L62	84±7.6		
	L5´	62±6		
	C9	58±7.6		
	C4	53±5.2		
	C5	53±2.9		
	L7	53±11		
Good adherence (10 - <50 adhered bacteria / Vero cell)	B3	42±8.5	18	35%
	L36	42±13		
	C8	33±2		
	B2b	33±2.3		
	L49	33±3.4		
	LS	30±4.4		
	L61	30±3.2		
	C7	26±0.9		
	B1	26±1		
	B2a	26±1.6		
	B10	26±0.86		
	L4	26±2.9		
	L38	26±1.9		
	L63	22±6		
	L39	21±3		
	L31	18±3		
	L46	17±3.5		
	L55	10±0.6		

Moderate adherence (1 - <10 adhered bacteria / Vero cell)	L33	9±0.6	15	29%
	L21	8±0.3		
	L32	7±0.6		
	L25	7±0.5		
	L37	5±0.44		
	L24	4±0.57		
	L50	4±0.72		
	S1	4±0.3		
	L22	3±0.28		
	B9	3±0.32		
	L27	3±0.4		
	L54	3±0.26		
	L5	3±0.17		
	L53	2±0.29		
	L5b	1±0.34		
Weak adherence (<1 adhered bacteria/ Vero cell)	B11	0.25±0.1	4	8%
	L13	0.15±0.05		
	L23	0.11±0.04		
	L9	0.1±0.015		
No adherence	L17	0.01±0.001	6	12%
	L15	0.01±0.0007		
	L26	0.01±0.003		
	C3	0.01±0.002		
	L28	0.01±0.0007		
	L48	0.01±0.001		

* Lactobacillus isolate with L codes were recovered from raw milk, C code from cheese, B codes from butter and that of S code from infant stool ** Equal to number of adhered bacteria / Vero cell.

The resistance of these *Lactobacillus* isolates to acid was tested by examining their survival in MRS (pH 3) at 37°C after 0, 1, 3 h and compared with control (MRS pH 6.2). According to the obtained results (**Figure 2**), 44 out of 46 *Lactobacillus* isolates were able to survive after exposure to pH 3 for 3 h. Furthermore, 28 isolates (C1, C4, C7, C8, C9, C10, B1, B2a, B3, B10, B11, L4, L5', L21, L24, L25, L27, L31,L39, L38, L37, L36, L33, L47, L49, L50, L53 & L55) were able to grow in MRS (PH 3) after 24 h incubation at 37°C giving turbidity comparable to that of the control when examined visually.

The survival of the 46 *Lactobacillus* isolates in MRS containing 0.3% bile salts (pH 6.2) at 37°C were examined at different time intervals (0, 1, 4 h) and compared with control (MRS pH 6.2). According to the obtained results (**Figure 3**), out of 46 *Lactobacillus* isolates, 33 isolates retained their viability after exposure to 0.3% bile salts for 4 h (reflecting the time spent in the small intestine). Furthermore, 15 isolates (C4, C7, C8,

C9, C10, B2a, B2b, B3, B10, B11, L36, L38, L39, L47 & L49) were able to grow in the presence of 0.3% bile salts after 24 h incubation at 37°C giving turbidity comparable to that of the control when examined visually.

After screening of all isolates, we obtained 32 *Lactobacillus* isolates tolerant to stomach acidity and intestinal bile and having adherence capabilities to mammalian cells as shown in Table (2).

Discussion

One of the most important criteria that a probiotic candidate should fulfill is its ability to adhere to the epithelial cells [15,19-20]. This property is important for colonization [21], pathogen exclusion, modulation of the immune system [22] and interaction with epithelial cells [23-24].
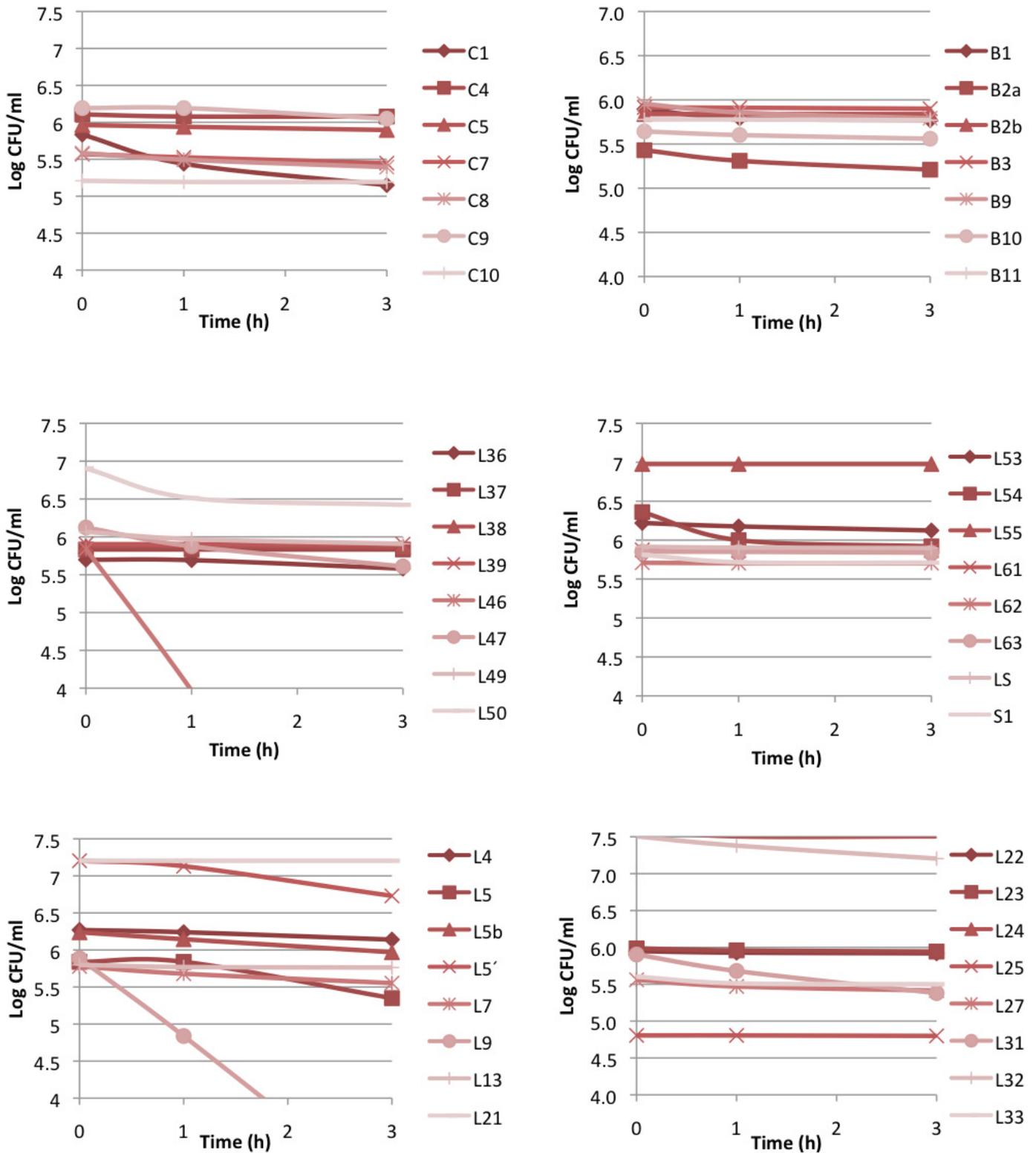
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Fig.2. Survival of *Lactobacillus* isolates in acidic medium (MRS pH 3)

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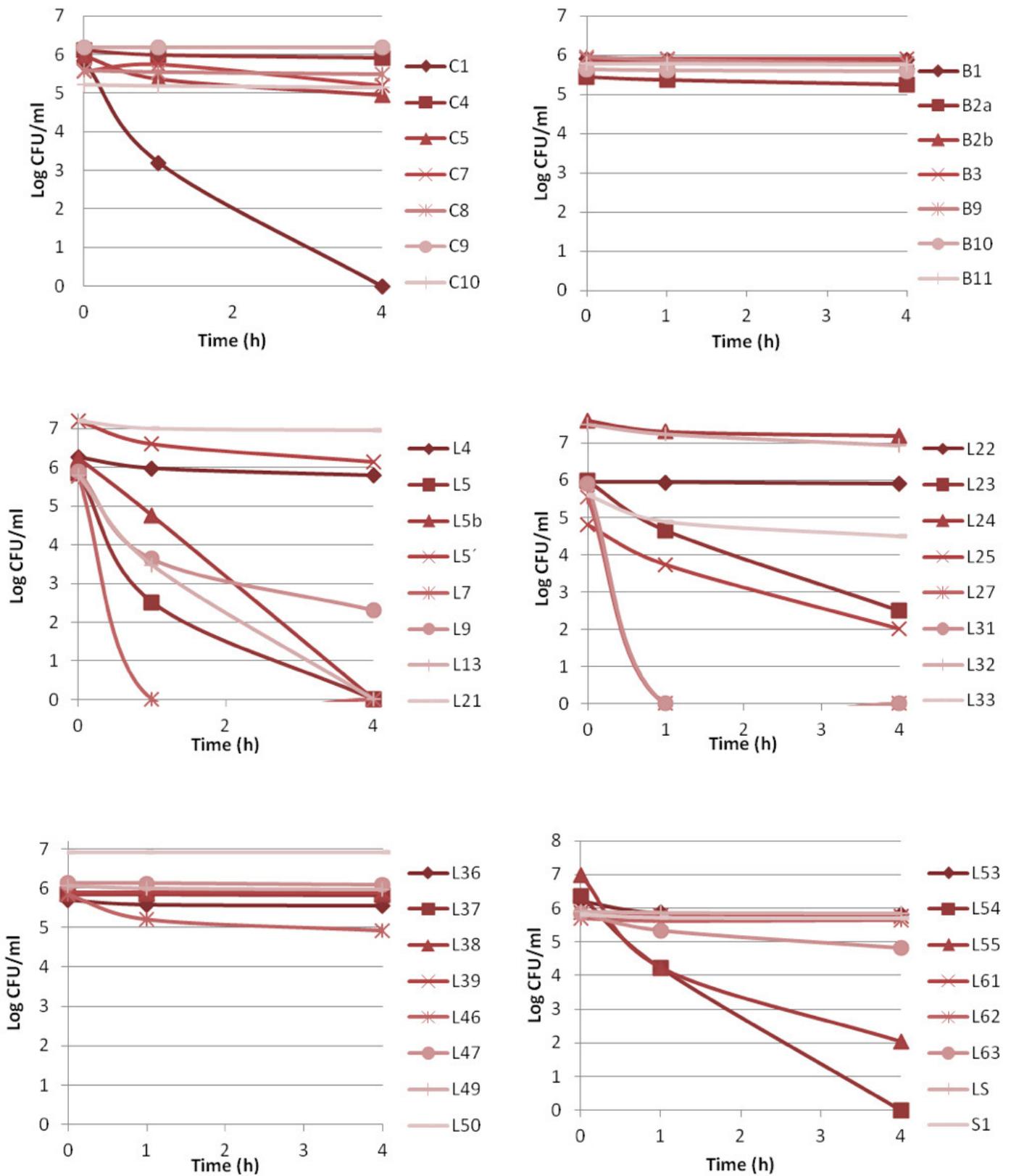
Fig.3. Survival of *Lactobacillus* isolates in presence of 0.3% bile salts at pH 6.2

Table 2. Probiotic characteristic of the tested *Lactobacillus* isolates

Isolate code	Adherence capacity	%Survival in MRS with:		Isolate code	Adherence capacity	%Survival in MRS with:	
		0.3% bile salts	pH 3			0.3% bile salts	pH 3
C4	53±5.2	63±7.1	94±5.7	L22	3±0.28	91±7.2	93±3
C5	53±2.9	9±5.8	86±9.1	L24	4±0.57	40±4	80±2.7
C7	26±0.9	43±4.1	75±6.8	L33	9±0.6	8±3.7	79±3
C8	33±2	81±6.4	65±4.4	L36	42±13	72±7.6	76±5.6
C9	58±7.6	100±0.7	72±2.7	L37	5±0.44	98±2.7	100±0.6
C10	84±1.4	86±6.1	96±3.3	L38	26±1.9	97±2.1	98±1
B1	26±1	96±1.1	75±7.6	L39	21±3	100±0.2	100±1
B2a	26±1.6	66±8.1	60±12.8	L47	167±22	92±2.5	31±3
B2b	33±2.3	99±0.8	100±0.4	L49	33±3.4	80±4.8	70±7.1
B3	42±8.5	100±0.5	97±1.1	L50	4±0.72	99±0.6	33±6.3
B9	3±0.32	67±4.7	69±3.8	L53	2±0.29	40±2.1	80±1.8
B10	26±0.86	89±2.8	83±3	L61	30±3.2	79±6.1	97±3.1
B11	0.25±0.1	96±2.7	98±2	L62	84±7.6	88±2	99±0.8
L4	26±2.9	33±6.3	74±4.2	L63	22±6	9±3.4	97±1.7
L5´	62±6	9±4.2	34±3.4	LS	30±4.4	87±3	96±1.5
L21	8±0.3	57±7	100±0.9	S1	4±0.3	78±4.5	79±4.1

A variety of *in vitro* model systems for routine adhesion experiments e.g. *Vero* cell [25-26], Caco-2 & HT-29 [27] were used. In the present study, *Vero* cell was used as a model for investigation of bacterial adhesion to the normal epithelial cells (not cancerous cells). The adherence capacities of the tested *Lactobacillus* isolates (52 isolates) to *Vero* cells varied greatly(**Figure 1** and **Table 1**), where nine *Lactobacillus* isolates (C1, C4, C5, C9, C10, L5', L7, L47and L62) showed high adherence (more than 50 adhered bacteria per *Vero* cell), eighteen *Lactobacillus* isolates (C7, C8, B1, B2a, B2b, B3, B10, L4, L31, L36, L38, L39, L49, LS, L61, L63, L 46 and L55) showed good adherence (10 to 50 adhered bacteria per *Vero* cell), fifteen *Lactobacillus* isolates (L33, L21, L32, L25, L37, L24, L50, S1, L22, B9, L27, L54, L5, L53 and L5b) showed moderate adherence (>1 adhered bacteria per *Vero* cell), four *Lactobacillus* isolates (B11, L13, L23 and L9) showed weak adherence (<1 adhered bacteria per *Vero* cell) and six *Lactobacillus* isolates (L17, L15, L26, C3, L28 and L48) showed very poor adherence to *Vero* cells. Many studies showed that adhesive properties are not a universal feature of *Lactobacillus* because it varies considerably between *Lactobacillus* strains [21,28].

It is worth noting that some *Lactobacillus* isolates recovered from either raw milk or dairy products exhibited adherence

to *Vero* cell higher than a representative intestinal *Lactobacillus* isolate (S1).

Several studies showed that lactobacilli have good capabilities to bind to different types of epithelial cells such as human vaginal epithelial cells, to cultured human carcinomal intestinal cell lines, to intestinal mucus, and to the components of the extracellular matrix (ECM) [28-41].

The adhesiveness of lactobacilli is strain-specific [8]. Several studies showed that the microbial adhesion process of lactobacilli includes passive forces, electrostatic interactions, hydrophobic steric forces, lipoteichoic acids; and specific structures [2,8,31, 42-45]. McGroarty identified by transmission electron microscopy fimbriae on vaginal strains of *L. rhamnosus, L. acidophilus, L. jensenii, L. casei,* and *L. fermentum*. Fimbriated lactobacilli, *in vitro*, adhered in significantly greater numbers to human vaginal epithelial cells than those nonfimbriated variants [46]. Chan *et al.* suggested that lipoteichoic acid participates in the adherence of lactobacilli to uroepithelial cells [29], whereas Reid *et al.* identified two adhesins, an extracellular, probably proteinaceous, and a trypsininsensitive cell wall adhesion [47]. Boris *et al.* showed that three strains of lactobacilli, *L. acidophilus, L. gasseri* and *L. jensenii* strongly adhered to vaginal epithelial cells *in vitro* [31].

However, several ways of adherence exist: in *L. acidophilus* and *L. gasseri* proteins and carbohydrates participate in the adherence, whereas *L. jensenii* adherence seems to depend on carbohydrates alone. Sillanpaa and his workers reported that the S-layer protein from *L. crispatus* strains is involved in their adhesion [48]. For strains of *L. plantarum*, isolated from the human gastrointestinal tract, an expression of a mannose binding adhesin could be shown [2]. These findings confirm the presumption that different strains of lactobacilli differ in their capacity and way of adherence.

After oral ingestion of probiotic bacteria, they encounter a number of human defense systems that are associated with secretions. These include gastric acid inducing a low pH in the stomach, and bile salts secreted into the luminal content in the proximal small intestine [49].

Adherence to epithelial cells, resistance to gastric acidity and resistance to bile salts are among the *in vitro* tests that are frequently suggested for the evaluation of the probiotic potential of a bacterial strain [50-51]. In the present study, the 6 *Lactobacillus* isolates with no adherence capacity were excluded and the remaining 46 isolates were selected to investigate their probiotic potential, which included their acid and bile tolerance and antagonistic activity against some human pathogens. The tolerance to stomach acidity was investigated by testing the survival of *Lactobacillus* isolates in MRS at pH 3 which mimic the stomach pH. The survival also examined at different time intervals which reflect the time spent by food in the stomach. The results showed that only two isolates out of 46 can't survive in acidic pH after exposure for 3 hours, while the remaining isolates showed acid tolerance expressed as survival percentage in acidic medium pH3 ranged between 30 – 100% survival (**Table 2**). These survival capabilities could be categorized into high survival percentage (75 – 100%), good survival percentage (50 - <75%), and moderate survival percentage (25 - <50%).

It is worth noting that the lowest survival percentage (21%) of the acid tolerant 44 *Lactobacillus* isolates didn't exceed one Log_{10} reduction of initial count. Furthermore, prolonged incubation in the acidic medium of pH3 was overcome by about 64% of the acid tolerant isolates and resulted in an increase in growth after 24h.

These results were in agreement with those obtained from previous similar *in vitro* studies, where *Lactobacillus* strains were able to retain their viability when exposed to pH values of 2–3 [10,50,52-56]. However, Schillinger *et al.* found that *Lactobacillus* strains isolated from various probiotic dairy products differed considerably in their resistance to acid in a simulated gastric juice [27].

Conway *et al.* and Ouwehand *et al.* reported that survival of ingested bacteria in the stomach obviously is influenced by the buffering capacity of food components [10, 57]. In this context, even strains not able to survive at pH 2 *in vitro* could exhibit substantial viability when consumed in a matrix of fermented milk, where milk substances may provide a protective matrix enhancing survival of bacteria in the gastric juice.

Resistance to bile salts is generally considered as an important criterion for probiotic selection, since it is an essential property for probiotic strains to survive the conditions in the small intestine. The tolerance to bile was investigated by testing the survival of 46 *Lactobacillus* isolates in MRS enriched with 0.3% bile salts which mimic the physiological bile concentration [18]. The survival also examined at different time intervals which reflect the time spent by food in the small intestine. The results showed that 33 (about 70%) *Lactobacillus* isolates were able to survive in presence of bile after 4 hours exposure and could be considered as bile tolerant. These bile tolerant isolates showed different survival capabilities in MRS containing 0.3% bile salts after 4 h (**Table 3**) ranged between (8 – 100%). It could be relatively categorized into high survival percentage (75 – 100%), good survival percentage (50 - <75%), moderate survival percentage (25 - <50%) and weak survival percentage (20 - <50%).It is worth noting that and as observed for acid tolerance, 15 *Lactobacillus* isolates were able to grow in the presence of 0.3% bile after 24 h incubation at 37°C. In a similar study it was found that different strains of *L. casei* were able to grow in the presence of 0.5% bile [58]. Also, it was observed that twelve *Lactobacillus* isolates can't survive in presence of 0.3% bile after 4 h, in spite of their acid tolerance (**Tables 2 & 3**).

Regarding acid and bile tolerance, the mechanisms involved in acid resistant of lactobacilli are not fully understood, but several studies detected some genes that are switched on under conditions simulating GIT. Bron and co-workers showed up regulation of stress proteins, cell envelope located proteins, and proteins involved in redox reactions after exposure of *Lactobacillus* strains to 0.1% porcine bile [59]. Many authors suggested that the resistance of *Lactobacillus* strains to the toxicity of bile salts in the duodenum may be attributed to bile salt hydrolytic (BSH) activities [60]. Conversely, other studies showed that resistance to bile salts and bile salt hydrolase activity are unrelated in lactobacilli [61]. Several *in vitro* studies showed alterations in the cell wall, presumably to protect the cell from the harsh conditions (acid and bile).

Interestingly and as observed in case of adherence, the results showed that the survival percentage of some *Lactobacillus* isolates recovered from dairy products after exposure to 0.3% bile salts was higher than that of *Lactobacillus* isolate S1 which is of intestinal origin. According to these findings,

Lactobacillus isolates recovered from dairy products could be able to colonize and survive the harsh conditions encountered during the passage through GIT efficiently comparable or even superior to intestinal lactobacilli.

In agreement with the present study, different strains of *L. plantarum* were found to show a high tolerance to the consecutive exposure to hydrochloric acid (pH 2.0) and bile salts. This was observed both for strains isolated from intestinal samples and for those isolated from fermented foods [55].

To conclude, in this study thirty two Lactobacillus isolates were found to possess desirable probiotic properties. These isolates are good candidates for further investigation in *in vitro* and *in vivo* studies for their potential health benefits and their application as novel Biotherapeutic agents.

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