Probiotic potential of novel strains of *Lactobacillus plantarum* Lp -1: *In vitro* studies

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Abstract: Five Lactobacillus strains isolated from vegetable and dairy products showed 99% similarity with *Lactobacillus plantarum* 1(Lp-1) using API –CHL 50 kit. Most of them proved to be sensitive to bacterial cell-wall inhibitors i.e. penicillin, ampicillin, amoxicillin and methicillin as studied by disc-diffusion method. These strains manifested profound tolerance to acidic-stress where Lp86 and Lp36 exhibited a good survival pattern at pH-2 for 4 hr retaining a survival count of 85% and 50%, respectively. A high survival of 85.7% was witnessed in Lp86 in presence of protease while Lp36 maintained 94.55% and 92.65% of population under the influence of enzyme pancreatin and pepsin. All the strains displayed marked tolerance against trypsin as the count did not drop below 77%. Absorbance and growth in terms of cfu/ml for bile-tolerance was examined for concentrations reflecting those in the GIT of humans, all the Lp-1 strains when grown with 1% bile showed a drop in the viable count by 1 log cycle i.e. from 10^{10} to 10^{9}cfu/ml. Fulfilling the above mentioned criteria these probiotic candidates displayed their capacity to reach the colon as viable metabolically active cells after successfully surviving under conditions similar to the gastrointestinal tract of humans. Upon examining the viability and stability of these probiotic candidates in most common foods serving as vehicle for probiotic delivery to the intestine, it was noticed that all the isolates tested sustained a probiotic approved number of 10^{7} cfu/ml for effective function as recommended by WHO, after a maximum storage for one month. Hence, it could be justified that the selected probiotic candidates possess prominent probiotic potential. Therefore, *L. plantarum* 1 strains could prove to be an efficient probiotic after further in vivo studies to explore its safety in human subjects.

Keywords: Probiotic and *Lactobacillus plantarum*.

INTRODUCTION

Lactic acid bacteria (LAB) are known to possess a versatile distribution in the environment and most of its species have proved to be efficient probiotics capable of surviving during gastric transit. They were well known as starter cultures for fermentation, enhancing the flavor and texture of foods (Mantzourani *et al.*, 2019). Until now, it has been established that a variety of Lactobacillus species successfully inhabit the gastrointestinal tract of humans as well as other animals which include *Lactobacillus acidophilus, Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus vaginalis, Lactobacillus delbrueckii, Lactobacillus casei, Lactobacillus salivarus* and function beneficially in maintaining the gut health (Sarmiento-Rubiano *et al.*, 2010). *Lactobacillus plantarum* is one of the species that normally resides in the human guts and have been utilized in industries for fermentation and preservation (Ramli *et al.*, 2012). Therefore, the LAB have been focused as important probiotics able to improve health; preventing many diseases including inflammatory-bowel disease and gastrointestinal infections (De Vries *et al.*, 2006). They have been valuably used for therapeutic purposes especially in lactose intolerance and cholesterol lowering effects (Yu *et al.*, 2013). The hypothesis proposed for this action could be the cholesterol assimilation by the bacteria, cholesterol binding to the bacterial wall and deconjugating bile acids by bilesalt hydrolase activity (Pereira and Gibson, 2002). *L. rhamnosus GG, L. casei Shirota and L. plantarum* PCA236 were also proved to be effective against Rotavirus (RV) and Transmissible gastroenteritis virus (Maragkoudakis *et al.*, 2010). On the basis of the above attributes, probiotic bacteria are described as "living microorganisms" capable of conferring health benefits to humans (Zmora *et al.*, 2018). Thus, current study presents in vitro attempts made to investigate the survival of indigenous *L. plantarum* 1 strains in human digestive tract to highlight their probiotic potential.

MATERIALS AND METHODS

Identification of probiotic LAB strains

Five LAB isolates were selected for this study. They were initially identified based on their metabolic profiles using API CHL 50 KIT (Bio Merieux) according to the manufacturer's protocol. Briefly, a heavy suspension of each culture was prepared in 5ml de Man Rogosa Sharpe medium (MRS) (Oxoid) corresponding to 0.5 McFarland index. Finally, 600µl of suspension was transferred to 10ml ampule of API CHL50 KIT medium following the kit manual. Biochemical profile was identified by using the Api web™ Identification software with database.

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Antibiotic susceptibility pattern

Antibiotic susceptibility was checked on MRS agar plates by disc-diffusion method. Culture suspensions were adjusted to 1 McFarland index (approx. $3x10^8$ cfu ml^{-1}) and spread on to the surface of agar plate using a sterile cotton swab. Antibiotic discs (Oxoid) were then placed on the surface of the plates and incubated at 37°C for 24 hr. The zones of inhibition were recorded and interpreted as sensitive, intermediate and resistant comparing with the values of National Committee for Clinical Laboratory Standards (NCCLS).

Survivability testing under conditions simulating the upper gastrointestinal tract

L. plantarum 1 strains were subjected to preliminary assessments for probiotics including their survivability during gastric transit. All the probiotic candidates were grown until exponential phase of growth and subjected to the following stresses:

Tolerance to acid

Tolerance to acid was assessed according to Maragkoudakis *et al*, (2010). The isolates were grown in MRS broth (50ml) for 18 hr to achieve a log-phase, centrifuged for 10 minutes at 10,000 r.p.m. Washed twice with sterile PBS (pH-7.4) and resuspended in the same. This cell suspension was adjusted to O.D 2.5 at A_{560} to obtain an initial 10^6 cfu/ml corresponding to 50 to 70 colonies in 30 ml MRS (pH 1, 2 or 3) at the beginning. A tube of MRS broth (pH-6.2) inoculated with a similar cell suspension as test served as a control. The tests and controls were incubated at 37°C for 4 hr reflecting the time spent by the food in the stomach. All tests were run in triplicates. Survival count was measured every hour as cfu/ml by spread plate technique. The survival percentage was calculated using the number of cells in the control as the total count.

Tolerance to enzymes

The effect of enzymes, pepsin, pancreatin, protease, trypsin and chymotrypsin on survival of probiotic *L. plantarum* strains was studied using methods described by Olejnik *et al*., 2005 and Maragkoudakis *et al*., 2010 with some modifications. Cultures were grown in 50 ml MRS, the enzyme solutions 3mg/ml were prepared in appropriate solvents; Pepsin (from porcine stomach mucosa , Sigma) in Tris-HCL (pH-2), Pancreatin (Mammalian origin, Fisher scientific) in PBS (pH-8), Trypsin (from Pancreas, Sigma-Aldrich, Germany), Chymotrypsin (MP Biomedical LLC., Solon, Ohio) and Protease (fungal origin, MP Biomedical LLC) in PBS (pH-7). Cultures were inoculated in 1.2 ml PBS supplemented with 0.6 ml of the respective enzyme solution, 0.2 (10^6cfu/ml) of cell suspension and incubated for 4 hr at 37°C. The survival percentage was enumerated at every hourly interval following the Standard spread plate technique. The number of colonies of 10^{-6} dilution was enumerated to calculate the survival percentage. All tests were run in triplicate.

$$\text{Survival \%} = \frac{\text{Number of colonies in test}}{\text{Number of colonies in control}} \times 100$$

Tolerance to bile

Bile salt tolerance (bovine B3883-25G, Sigma) was investigated by estimating the survival percentage of Lp-1 strains in presence of 0.5 and 1% bile in MRS broth following a method as described by (Maragkoudakis *et al*., 2010) by inoculating of 2% v/v of (10^6cfu/ml). Tolerance to bile was also assessed by recording the change in absorbance at A_{560} after every 30 minutes for 5 hr, representing the time spent by the food to get digested. For this purpose, 20ml MRS broth supplemented with 0.5% and 1% bile was inoculated with 1% cells (matched with 0.5 McFarland's index) starting with an initial absorbance of 0.1-0.2 a change in the growth pattern was determined.

Survival of probiotic strains in different probiotic vehicle foods

Whole pasteurized milk (100ml; pH7) with 16.5% skimmed milk was treated at 90˚C for 30 minutes and seeded with (1%) 10^8 cfu/ml (washed twice) colony forming units were determined on the MRS agar plates at the start, after 24 and 48 hr or until a pH of 4.8 was achieved.

Commercially prepared yogurt was used to assess the survival of selected probiotic candidates. In order to avoid the hindrance of starter culture of the commercial yogurt with test probiotic strain, the yogurt was heat treated at 91˚C for 40-60 seconds. An aliquot of heat-treated yogurt (9 grams) was transferred to sterile test bottles and inoculated with 10^8 cfu of the test strains per ml and incubated at 4˚C leaving no space for air to create microaerophilic environment. The number of cells was determined at 0 minutes, 48 hr and then after every week for up to a month.

Freshly extracted orange juice was filtered, heated at 90˚C in a water-bath for 5 minutes and sterile culture bottles were filled with 7.2 ml of orange juice (pH-5) inoculated with 0.8ml (10^8 cfu/ml) of washed cell-suspension of test probiotic candidates and incubated in a refrigerator. This was followed by the determination cfu/ml after zero min, 5, 10, 15, 20, 25 and 30 days. Enumeration of cells plated at 10^{-6} dilution was done according to the WHO approved number of probiotic organisms in any food supplemented with probiotic strains simultaneously also recording the drop in pH at periodic interval.

RESULTS

Biochemical identification of LAB isolates

All the five isolates belonged to the genus *Lactobacillus* and showed 99% similarities with *Lactobacillus*

plantarum 1 designated as, *Lactobacillus plantarum* 86 (Lp86), *Lactobacillus plantarum* 52 (Lp52), *Lactobacillus plantarum* 36 (Lp36), *Lactobacillus plantarum* 32 (Lp32), *Lactobacillus plantarum* 07 (Lp07).

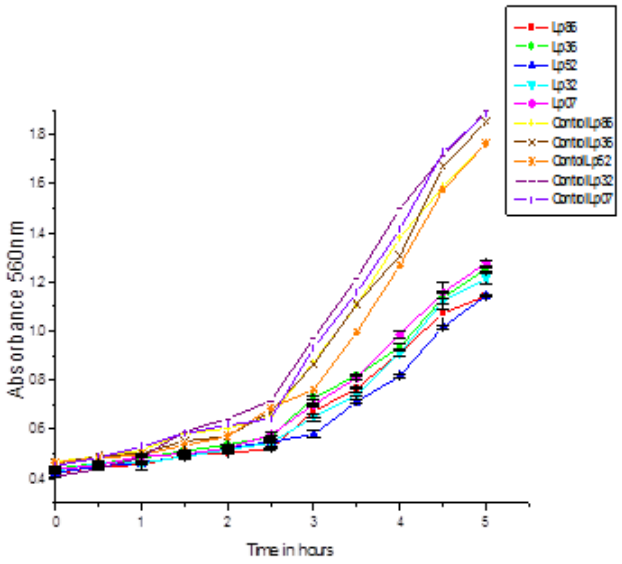

Fig. 1: Survivability of *L. plantarum* 1 Probiotic candidates in presence of 0.5% bile terms of absorbance with control (MRS without bile).

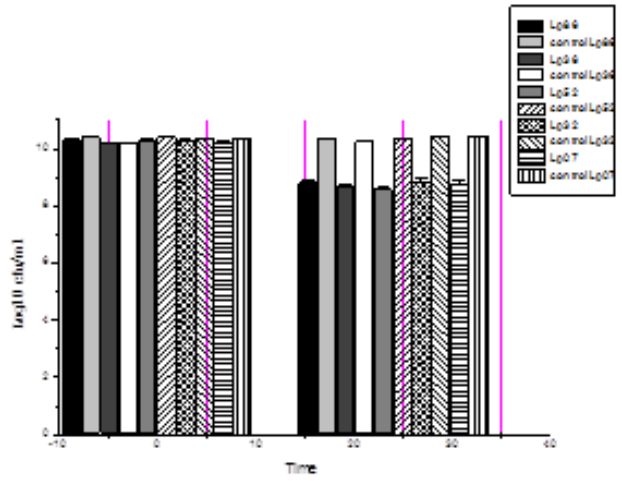

Fig. 2: Survivability of *L. plantarum* 1 probiotic candidates in presence of 0.5% bile in terms of log 10 of cfu/ml with their respective controls (MRS without bile).

Antibiotic susceptibility of LAB isolates

All the five strains of *L. plantarum* 1 were resistant to tetracycline, gentamycin, kanamycin, metronidazole and Cefoxitin. However, susceptibility to penicillin, methicillin ampicillin, chloramphenicol and Amoxycillin was noticed (table 1).

Tolerance to acid

The data suggest that the strain Lp32 had shown a very high degree of survival at highly acidic (pH-1) of 40.16% for 1 hour followed by Lp86, Lp36 and Lp52 with a survival of 7%, 1.66% and 0.93 %, respectively. Nonetheless, the growth ceased completely after 1 hour. Furthermore, Lp07 was highly sensitive to pH-1 showing no growth (table 2).

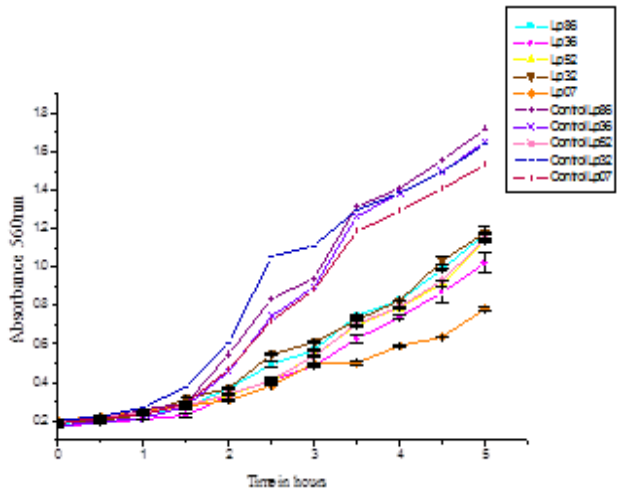

Fig. 3: Survivability of *L. plantarum* 1 Probiotic candidates in presence of 1%bile in terms of absorbance with their respective controls (MRS without bile).

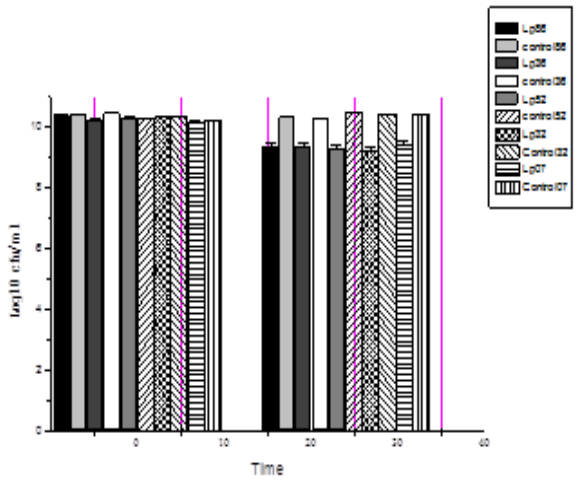

Fig. 4: Survivability pattern of *L. plantarum* 1 probiotic candidates in presence of 1% bile in terms of log10 of cfu/ml with their respective controls (MRS without bile).

Lp86 and Lp36 exhibited a good survivability at pH-2 for 4 hr with a population density of 85% and 50%, respectively. On the contrary, Lp32 and Lp52 survived only in the first hour while Lp07 being the most sensitive didn't show any growth even after the first hour. All the isolates showed a survival at pH-3 during the entire incubation period. Lp36 showed 88% survival in the first hour and 91% in the third hour and retained 50% viability at the end of experiment. This was followed by the others showing population density slightly less than 50%.

Tolerance to enzymes

Lactobacillus plantarum 86 (Lp86) demonstrated the maximum tolerance to 3mg/ml of protease with a survival of population of 85.71% after 4 hr while Lp36, Lp52, Lp32 and Lp07 also survived for not less than 63.46% (table 3).

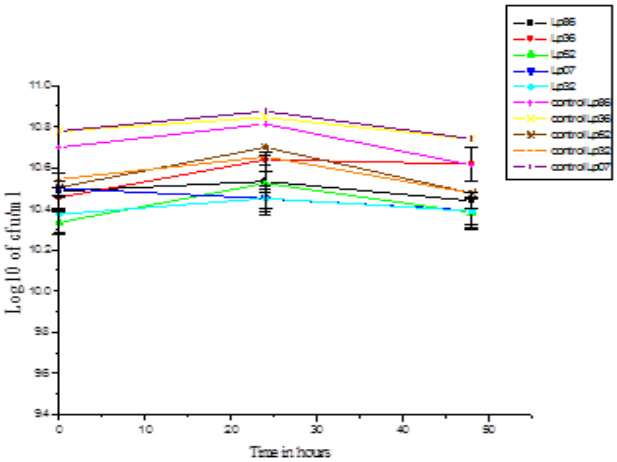

Fig. 5: Survivability of *L. plantarum* 1 probiotic candidates in milk in terms log 10 cfu/ml with their respective controls (MRS Broth).

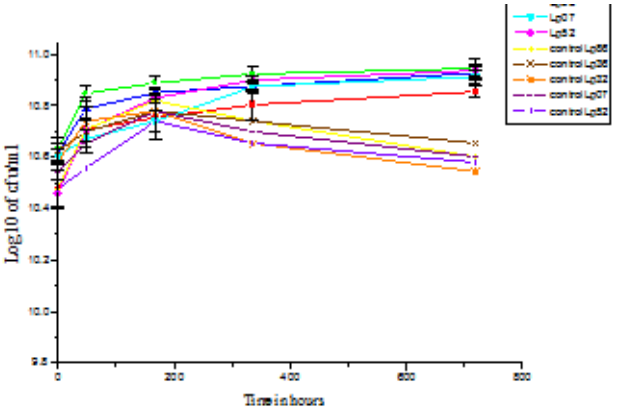

Fig. 6: Survival of *L. plantarum* 1 probiotic candidates in yogurt for 720 hr (1month) and in MRS as control in terms of log 10 of cfu/ml.

A higher degree of survivability with slight reduction in cell count (~3.45%) was noted after 4 hr of incubation in strain Lp36 in presence of pancreatin, while rest of the showed a survival of 74.54% for Lp86, 71.98% for Lp07. The strains, Lp32 and Lp52 exhibited only 50% viability after 4 hr (table 3). Lp36 showed resistance to pepsin with a 92.65% survival followed by Lp07 with 91.22% survival. It is interesting to note that the rate of survival of Lp32, Lp52, Lp86 never fall <69% even after 4 hr of incubation (table 3).

The data about the effect of Chymotrypsin (3mg/ml) on the probiotic strains shows that the viable count of Lp07 and Lp86 reached up to 93.51% and 81.55% whereas Lp52, Lp36 and Lp32 showed a slight decrease in number during the incubation but never dropped below 64 %.

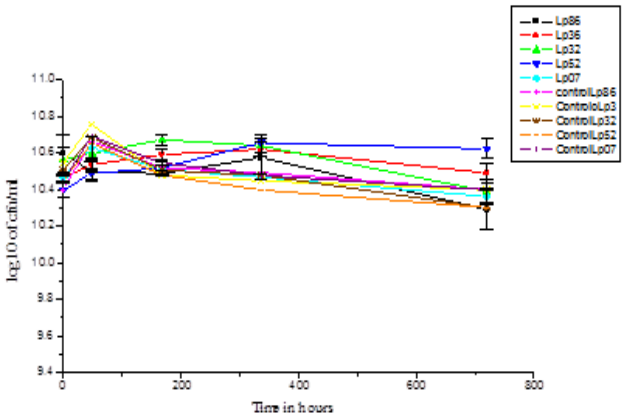

Fig. 7: Survival of *L. plantarum* 1 probiotic candidates in orange juice for 1 month (720hr) and in control (MRS broth) in terms of log of cfu/ml.

It is clear from the data that probiotic candidates showed more resistance to trypsin (3mg/ml) compared to other digestive enzymes used in this study. Where the minimum count recorded after 4 hr was 77.18%, while the most tolerant strains being Lp07 with 92.53%, Lp36 with 91.98%, Lp86 with 90% and Lp52 with 88.79% population.

Tolerance to bile

All the five probiotic candidate strains grew well for 5 hr in presence of 0.5% bile, as determined spectrophotometrically (fig. 1). It seems there was not much difference in absorbance of control and test in the later hr of assessment which indicates tolerance of these strains to bile. The resistance pattern of all the five probiotic strains of *L. plantarum* was quite similar reflecting the ability of these strains to survive even in presence of 1 % bile which is several folds higher than the concentration found in stomach during food digestion (fig. 3). The resistance pattern, in terms of viability reflects that the drop in the viable count was only 1 log cycle i.e. from 10^{10}- 10^{9}cfu/ml (fig. 4).

Survival in probiotic vehicle foods

All the strains of *L. plantarum* selected in the present study showed significant survival for 48 hr in milk or until the pH reached 4.8. Amongst all the strains, Lp36 demonstrated maximum survival i.e. 10.6 $\log^{10}$ cfu/ml at the end of incubation. Lp52 also sustained a count not less than the initial inoculum (10.5 $\log^{10}$ cfu/ml). The survival of Lp32 and Lp86 was not much different from Lp52 as the count remained the same throughout the test. On the other hand, the Lp07 count showed an insignificant drop in the initial number to 10.4 $\log^{10}$cfu/ml, being sufficient for a probiotic number, as approved by WHO/FAO. The pattern of survival of all the *L. plantarum* 1 probiotic strains in milk resembled that in control (MRS) (fig. 5).

Table 1: Antibiotic susceptibility pattern of *L. plantarum* 1 strains (By Disc Diffusion)

ANTIBIOTICS	Lp07	Lp32	Lp36	Lp52	Lp86
Penicillin (10 units)	22 (S)	25 (S)	18 (S)	23 (S)	24 (S)
Erythromycin(15µg)	19 (I)	23 (S)	17 (I)	17 (I)	21 (I)
Tetracycline (30µg)	13 (R)	17 (R)	10 (R)	15 (I)	18 (R)
Ampicillin (25µg)	25(S)	40 (S)	32(S)	45(S)	37(S)
Ampicillin (10µg)	23 (S)	36 (S)	28 (S)	42 (S)	32 (S)
Gentamycin(120µg)	11(R)	11(R)	12(R)	14(R)	11(R)
Gentamycin(10µg)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)
Kanamycin(30µg)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)
Chloramphenicol (30µg)	19 (S)	20 (S)	20 (S)	20 (S)	21 (S)
Amoxycillin(25µg)	24 (S)	30 (S)	23 (S)	28 (S)	30 (S)
Amoxycillin(10µg)	27 (S)	25 (S)	22 (S)	24 (S)	29 (S)
Methicillin(10µg)	13(R)	12(R)	11(R)	14(R)	12(R)
Methicillin(5µg)	7 (R)	7 (R)	9 (R)	10(R)	7(R)
Metronidazole(50µg)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)
Cefoxitin(30µg)	ND	11 (R)	0 (R)	11 (R)	7 (S)

Table 2: Survival percentage of *L. plantarum* 1 strains at acidic pH values

L. plantarum strains	Survival %, ± SD				Survival %, ± SD				Survival %, ± SD			
	PH-1				PH-2				PH-3			
	1hr	2hr	3hr	4hr	1hr	2hr	3hr	4hr	1hr	2hr	3hr	4hr
Lp86	7.0 ±1.5	0 ±0.0	0 ±0.0	0 ±0.0	45 ±1.5	60 ±1.5	80 ±2.0	50 ±5.5	51 ±2.0	67 ±1.5	84 ±1.5	49.66 ±1.5
Lp36	1.66 ±1.5	0 ±0.0	0 ±0.0	0 ±0.0	86.16 ±2.5	98.50 ±3.6	96.81 ±4.0	85.83± 2.5	88.33 ±10.2	67.66 ±15.1	91.66 ±10.0	50.83 ±11.6
Lp07	0 ±0.0	0 ±0.0	0 ±0.0	0 ±0.0	0 ±0.0	0 ±0.0	0 ±0.0	0 ±0.0	59.2 ±10.4	54.99 ±6.0	96.15 ±10.0	41.66 ±10
Lp52	0.93 ±0.5	0.55 ±1.5	0 ±0.0	0 ±0.0	1.6 ±2.0	0 ±0.0	0 ±0.0	0 ±0.0	57.33 ±7.6	37.66 ±10.5	25.87 ±2.8	38.57 ±3.6
Lp32	40.1 ±6	0 ±0.0	0 ±0.0	0 ±0.0	79.16 ±7.6	0 ±0.0	0 ±0.0	82.66 ±6.1	63.80 ±3.2	50.11 ±10.0	50.11 ±10.0	27.0 ±7.5

Table 3: Tolerance of *Lactobacillus plantarum* 1 strains to enzymes

L. plantarum strains	Time in Hours	Enzymes				
		Protease 3 mg/ml	Pepsin 3 mg/ml	Pancreatin 3 mg/ml	Trypsin 3 mg/ml	Chymotrypsin 3 mg/ml
		SUR % ± SD	SUR % ± SD	SUR % ± SD	SUR % ± SD	SUR % ± SD
Lp07	1 hr	93.33±1.7	98.44±1.1	90.47±6.5	96.3±2.51	95.54±2.08
	2hr	83.33±1.5	97.12±1.1	90±5.5	92.73±2.30	83.32±1.15
	3hr	78.33±2.0	97.94±1.5	9.33±5.6	93.63±3.51	86.18±2.52
	4 hr	98.24±1.0	91.22±2.0	71.98±4.1	92.53±2.64	93.51±3.05
Lp32	1 hr	90.22±2.5	97.03±1.5	64.21±6.2	84.21±5.0	92.22±3.05
	2hr	77.29±2.0	80.60±3.0	92.92±8.1	86.33±3.21	86.07±1.52
	3hr	95.0. ±2.0	68.75±2.0	89.62±4.9	81.96±5.50	68.83±3.05
	4 hr	97.12±1.5	88.00±2.6	83.33±4.4	77.18±1.15	79.34±3.78
Lp36	1 hr	62.05±3.2	94.30±1.5	55.37±4.5	95.73±4.93	96.79±3.78
	2hr	74.81±3.0	93.33±1.5	87.96±4.3	94.84±5.03	94.8±2.64
	3hr	100± 5.2	87.35±2.0	86.78±6.1	91.10±1.52	94.11±2.0
	4 hr	63.46±3.0	92.65±2.5	94.55±2.5	91.98±6.80	75.86±4.16
Lp52	1 hr	72.91±0.5	95.59±1.5	87.75±6.5	95.48±2.08	95.45±3.60
	2hr	98.92±1.5	95.75±1.5	84.09±3.6	92.09±2.517	72.86±3.055
	3hr	96.56±2.08	93.93±2.0	86.06±2.5	89.11±6.11	71.57±2.0
	4 hr	94.35±2.08	71.75±2.5	51.33±4.0	88.79±1.52	64.28±2.646

The survival of these probiotic candidates was quite high in yogurt, as the viability increased with the passage of time until one month. Whereas, in controls (MRS), these strains showed a decrease after 1 week of incubation. Maximum survival and growth exhibited by strain Lp36 showing an increase in initial numbers from 10 to 10.9 log^{10}cfu/ml followed by rest of the strains with minor variations at the completion of one month of storage duration (fig. 6).

The viability of all the *L. plantarum* 1 probiotic candidates remained unaffected during the entire period of storage in orange juice. An increase was noticed till the 2^{nd} week showing a continued survival in the following weeks whereas a decrease was noticed at the end of one month (fig. 7).

DISCUSSION

This study focuses on evaluating some probiotic attributes of the selected strains of *L. plantarum* Lp 1. Primarily, the *Lactobacillus* strains demonstrating the ability of bioactive agent production were identified up to species level on the basis of their biochemical profiles, all showed 99% identity with *L. plantarum* 1 species.

In vitro testing of *L. plantarum* strains was conducted as per guidelines of FAO/WHO (Hotel and Cordoba, 2001). One of the essential criteria for probiotic candidates was their potential of survival under adverse conditions, mimicking the human digestive tract which is the mandatory requirement to render its functional benefits as probiotic to the host. *L. plantarum* strains have displayed broad-ranged beneficial traits, which could be exploited while designing new probiotic supplemented fermented foods (Zago *et al.*, 2011). It is of utmost importance that a probiotic, after ingestion withstands, the strong acidic-environment of stomach, the action of digestive enzymes and survives at high concentrations of bile during the initial hr of digestion (Turchi *et al.*, 2013; Ru *et al.*, 2019).

The susceptibility profiles of the selected *L. plantarum* strains showed sensitivity to the cell-wall inhibitors often used in therapy i.e. penicillin, ampicillin, amoxicillin and methicillin which makes it a valuable trait (Yu *et al.*, 2013). All *L. plantarum* strains under study, demonstrated resistance to the second generation cephalosporin, cefoxitin, this may be a function of cell-wall impermeability (Charteris *et al.*, 1998). On the contrary in one such study, the disadvantages of resistance of *L. plantarum* species to tetracycline and erythromycin was demonstrated by the plasmid transfer to *Enterococcus faecalis*, this may lead to an eventual transfer of resistance to human pathogens (Jacobsen *et al.*, 2007). The resistance of Bifidobacteria to gentamycin, kanamycin, metronidazole and sensitivity to penicillin G, tetracycline is in lines with the present study (Moubareck *et al.*, 2005). Although Bifidobacteria are more susceptible to antibiotics than Lactobacillus but *L. plantarum* strains, under consideration, may also serve as better probiotic candidates being as safe as Bifidobacteria.

The survival of Lp36 and Lp86 at extreme acidic pH-1 and pH-2 indicates the possibility of successful transit in the human stomach even under fasting condition. On the other hands two strains of *L. plantarum* i.e. Lp52 and Lp07 seemed sensitive to such low pH but exhibited a better survival at pH-3. This finding correlates very well with Wang *et al*, 2011, where the cfu of *L. plantarum* isolated from weaning piglets decreased rapidly at pH-2 but survived at pH-3. Since all five *L. plantarum* strains retained almost 50% population during the entire digestion period. Hence, we can suggest these strains have ample chance to reach the intestine in high numbers in order to function effectively as probiotic. Reports of acid tolerance by Lactobacillus species are often documented and our results are quite in accordance to many such studies. Another similar research reported thirty Lactobacillus species including *L. acidophilus, L. plantarum, L. paracasei, L. rhamnosus*, resisting pH-3 for 3 hr but some showed a loss in viability at pH-1 only within an hour (Maragkoudakis *et al.*, 2010). *Lactobacillus plantarum* in a similar study exhibited good survival at pH-2 and 3 for 2-6 hr although a slight decrease in number was noticed with the increase in incubation time and the cell population reduced to less than 50% at pH-2 (Mourad and Nour-Eddine, 2006). On the other hand, the tolerance of LAB to this biological barrier is also reported in simulated gastric juice where the viability losses were in orders of 6.0 log cycles at pH-2 and by 3-4 log cycles at pH-3, where *L. acidophilus* proved to be the most tolerant to extreme acidic pH (Vinderola and Reinheimer, 2003). In the present study, all the tested *L. plantarum* 1 probiotic candidates retained their viability in the presence of all the enzymes tested, for the complete digestive period maintaining more than 50% viability. Thus, the significant survival rate of these probiotic candidates assures that their growth and survival will remain unaffected by these enzymes during gastric transit. The amazing enzyme tolerance function displayed by *L. plantarum* has been reported due to the presence of an (STp) peptide having serine and threonine amino acid in abundance which lacks protease cleavage site (Bernardo *et al.*, 2012).

The highest concentrations of 1% bile used in our study did not completely inhibit the growth of any of the tested *L. plantarum* 1 strains for 24 hr. Although, a drop in viability was quite significant after 24 hr which supports the unanimous fact often published in various reports that for efficient functioning of a probiotic, a continuous uptake is required. A similar investigation on 11 *L. plantarum* strains revealed that all resisted 2 % bile giving a minimum viable count of 11% to a maximum of 65%

after 24 hr (Mourad and Nour-Eddine, 2006). On the other hand the survival behavior of Bifidobacteria differed widely with Lactobacillus species showing less than 50% survival with 1% bile (Delgado *et al.*, 2008). The inhibition by bile often shows a vast contrast in in vitro and in vivo results, as the bile secreted in human contains lipid emulsifiers, sodium cholate, sodium deoxycholate and chenodeoxycholate which inhibit the bacterial cell by disrupting the membrane but still this inhibition is far less deleterious than the bile added in bacterial culture media (Olejnik *et al.*, 2005). Probiotic studies reported on *L. plantarum* Lp9 showed a pronounced survival at bile concentration 1.5-2.0% which are several folds higher than the normal physiological concentrations of bile in human stomach (Kaushik *et al.*, 2009).

Studies on survivability of the *L. plantarum* 1 probiotic candidates under condition similar to the GIT helped us in investigating the survival of these strains in some of the most common foods used as carriers of probiotics. All of them showed a good survival in milk for 48 hr with slight increase in initial numbers indicating multiplication in the carrier food which will be an important attribute for these probiotics to reach in maximum numbers in the intestine. In many studies, the survival of *L. casei* strain Shirota (LcS) in milk have been established following which researchers have assessed its potential as a probiotic in healthy volunteers for the relief of constipation where the ingestion of *L. casei* 10^9 cfu/ml for 2 weeks maintained a number as high as $\geq 10^7$ in the stools which proves that it reached the intestine improving the bowel movement (Matsumoto *et al.*, 2006) Hence, the data of our present study for *L. plantarum* 1 probiotic strains survival in milk supports well for its incorporation as a probiotic in milk for human consumption for the alleviation of symptoms of constipation after essential safety considerations.

All the tested *L. plantarum* 1 strains showed an unaffected viability pattern when stored with yogurt at refrigeration temperature for an extended period of 30 days maintaining a probiotic approved number of 10^7cfu/ml for effective function after human consumption. Moreover, an increase in viable count was witnessed proving the ability of these strains to multiply at refrigeration temperatures during prolonged storage in yogurt. On the contrary, many reports deciphered a decrease in the viable probiotic count in yogurt below the significant probiotic concentration of 10^6cfu/g of a product. In such cases they managed to improve their viability by adding micronutrients including amino acids and peptides, by applying microencapsulation and selecting acid and bile tolerant strains for probiotic function (Shah and Ravula, 2000). Some researchers made asymptomatic volunteers to consume yogurt supplemented with *Lactobacillus acidophilus* and *Bifidobacteria lactis* found a suppression of *H. pylori* after 6 weeks of continuous intake (Wang *et al.*, 2011).

In fruit juice (orange) *L. plantarum* 1 candidates were able to maintain a significant viability recommended for a probiotic in WHO/FAO reports (2002) for an extended period of one month. The viability was not reduced in spite of the continuous agitation and opening of the bottles for enumeration at regular intervals. A similar data of a study on *L. rhamnosus* as probiotic in apple based fruit juice have reported the survival for more than a week where quite promising results were achieved under the conditions kept as those prevailing in consumers' homes i.e. temperature fluctuation and a common practice of opening of the juice pack for several times (Champagne *et al.*, 2008).

Analyzing the resistance pattern of our probiotic candidates with different parameters, it was concluded that the response of each candidate varied with different criteria used for assessment. It has now become a known fact that acid resistant strains of LAB are considered best for elaboration of probiotic products as this property guarantees its survival and passage through the human intestine (Moreno *et al.*, 2006). Hence, Lp36 and Lp86 showing the maximum resistance demonstrated high viability in dairy products and juices serving as suitable vehicle for these probiotics.

CONCLUSION

Lactobacillus plantarum 1(Lp-1) strains tested in the present study proved to possess probiotic properties being able to adapt stress conditions similar to the gastrointestinal tract. Their potential to function as an efficient probiotic can also be supported by their prolonged survival in milk, yogurt and fruit juice which are the primary vehicles for probiotic consumption by humans. To accept these probiotic candidates for commercial use further on, *in vivo* studies must be conducted to see their beneficial behavior in humans.

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