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EFFECT OF BILE SALT AND pH ON SURVIVAL AND BACTERIOCIN PRODUCTION BY *LACTOBACILLUS ACIDOPHILUS*

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Abstract – The present study focused on the effect of bile salt and pH on the survival and bacteriocin production by five isolates of *L.acidophilus*. The percentage reduction at different bile salt concentration and different pH with respect to time intervals were found to be significant. The result obtained indicated that all the five *L.acidophilus* isolates produced bacteriocins at different concentration of bile salt and at different pH, and showed varying zone of inhibition against the tested food spoilage and common enteric pathogenic bacteria. On the basis of present data it can be concluded that all the *L.acidophilus* isolates showed variable susceptibility for all the tested antibiotics and also comes in multi drug resistant (MDR) category. Further it can be concluded that all the *L.acidophilus* isolates fulfil basic criterion expected from probiotic strains, *i.e.* are capable of surviving in the *in vitro* conditions of the GIT at low pH and in the presence of the bile salts, can act as probiotic, and are helpful in the improvement of our intestinal flora. The verified antimicrobial activity of probiotic supports the development of this functional food as to the key improvement of the health of the consumers and use as food preservatives contents in different packed food products.

INTRODUCTION

Lactic acid bacteria which include genus *Lactobacillus*, are the most prevalently administered probiotic bacteria (Brashears *et al.*, 2003). Lactic acid bacteria (LAB) occur naturally in several raw materials like milk, meat and flour used to produce foods (Rodriguez, 1996). Lactic acid bacteria, mainly *Lactobacilli* and *Bifidobacteria*, may have several therapeutic functions, including antimicrobial activity, anticholesterol activity, improved lactose utilization, and anticarcinogenic activity (Chou and Weimer, 1999). Lactic acid bacteria are considered as a major group of probiotic bacteria. The probiotic concept has been defined by Fuller in 1989 that means "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". The probiotics are microbial cell preparations or components of microbial cells that have a beneficial effects on the health and well-being of the host (Salminen *et al.*, 1996). Another recent definition was by Schrezenmeir and De Vrese (2001) who defined probiotics as viable microbial food supplements which beneficially influence the health of the host. Several *Lactobacilli*, *Lactococci* and *Bifidobacteria* are known to be health benefiting bacteria (Rolfe, 2000; Tuohy *et al.*, 2003). LAB constitutes an integral part of the healthy gastrointestinal (GI) microecology and is involved in the host metabolism (Fernandes *et al.*, 1988). Fermentation has been identified as a mechanism of probiotics (Gibson and Fuller, 2000). Lactic acid bacteria are useful for reduced lactose intolerance, alleviation of some diarrheas, lowered blood cholesterol, increased immune response and prevention of cancer (Marteau and Rambaud, 1996). Lactic acid bacteria along with other gut microbiota utilizes various substrates like biogenic amines, lactose,and allergenic compounds into short chain fatty acids along with other organic acids and gases (Fuller, 1989). LAB synthesizes enzymes, vitamins, antioxidants and bacteriocin (Knorr, 1998). LAB capable of producing antimicrobial peptides is used in a probiotic manner as food preservatives as well as health- promoting agents for humans (O'Sullivan *et al.*, 2002).

It has been observed that feed supplemented with few probiotic bacteria significantly reduce the numbers of enteropathogenic bacteria found in cattle rumen and feces (Brashears *et al.*, 2003). Lactic

acid bacteria (LAB) commonly used as starter cultures are known to produce antimicrobial substance such as bacteriocins (Avonts *et al.,* 2004). For probiotic purpose bacteriocins are generally produced by LAB strains in the product (Dunne and Shanahan, 2003). *L. acidophilus* has received more attention and has been the subject of much research due to its ability to produce antimicrobial agents against other bacteria. Bacteriocins are antimicrobial proteins or peptides, which are bacteriocidal to other, usually closely related bacteria (Du-Toit *et al.,* 1998). The ability of*L.acidophilus* to prevent pathogenic bacteria proliferation has been well documented. Bogovic-Matijasic and Rogelj (2000) isolated two bacteriocin of *L.acidophilus* which had an antagonistic effect against *Bacillus cereus, Clostridium sp*. and *Listeria innocua*. Zamfir *et al.* (2000) showed antibacterial activity against *E.coli* and *Salmonella* by *L.acidophilus*. *L.acidophilus* has the capacity to produce numerous metabolites that kill pathogenic bacteria (Gukasian *et al.,* 2002). *L.acidophilus* and *L.casei* are frequently used as probiotic agents (Kullen *et al.,* 2000). In similar studies, *L.acidophilus* isolated from dairy product have been nominated as the best bacteriocin producing strain (Avonts *et al.,* 2004). The criteria for selection as probiotic LAB include, human origin, safety, viability/activity in delivery vehicles, resistance to acid and bile, adherence to gut epithelial tissue, ability to colonise the gastro intestinal tract, production of antimicrobial substances, ability to stimulate a host immune response and the ability to influence metabolic activities such as vitamin production, cholesterol assimulation and lactose activity (Salminen *et al.,* 1996). Many factors may promote translocation of intestinal bacteria, such as intestinal mucosal injury, abnormal intestinal bacterial microbiota (Berg, 1995) previous antibiotic treatment, complications from Acquired Immunodeficiency Syndrom (AIDS) and prior hospitalization and surgery (Cooper *et al.,* 1998). The development of novel approaches in food (Luoma *et al.,* 2001) and in pharmaceutoclinical therapies (Steidler *et al.,* 2003) allow broadening the potential for using lactic acid bacteria in food and pharmacology (Renault, 2002). Therefore now a days there are increased tendency to incorporate probiotic organisms to improve the therapeutic value of various food products. Probiotic LAB are required to survive during gastrointestinal transit, recognise as safe and well grown in milk. Some LAB strains can colonize the gastrointestinal tract, and that is important factor to balance the intestinal microflora. To achieve this colonization, these bacteria must overcome biological barriers that include acid in the stomach and bile salts (Tsuda *et al.,* 2007). To characterize any organisms under the probiotic categories there are certain parameters as organism should be resistant for intestinal pH, adhere to the intestinal mucosa of the host, be nontoxic and nonpathogenic to host, exert a beneficial effect on the host, remain viable for a long time and with stand HCl in the host stomach and bile in the small intestine. There are several reports that suggests that pH (Murthy *et al.,* 2000; Goderska and Czarnecki, 2007) and different concentration of bile salt (Gopal *et al.,* 1996; Tsuda *et al.,* 2007) stimulate the survival and bacteriocin production by *L.acidophilus*. Therefore in the present study few *L.acidophilus* isolates were selected and screened for some of the above mentioned characteristics to place them under the category of probiotic organism.

MATERIALS AND METHODS

Study materials

Test organisms

In the present study five *L. acidophilus* isolates (LA1, LA2, LA3, LA4 and LA5) pre-isolated from curd were used. The antagonistic activity of *L. acidophilus* isolates were screened against following pathogens.

1. *E.coli (MCCB0017)*
2. *S.aureus (MCCB0046)*
3. *S.typhi (MCCB0021)*
4. *B.cereus (MCCB0006)*

Collection and maintenance of cultures

2 (a) Collection of *Lactobacillus acidophilus* isolates

For the study, the isolates of *L.acidophilus* were obtained from Microbial Culture Collection Bank, Department of Microbiology and Microbial Technology, College of Biotechnology and Allied Sciences, Allahabad Agricultural Institute -Deemed University.

2(b) Maintenance of *Lactobacillus acidophilus* isolates

The *L.acidophilus* isolates were maintained by successive transfer in MRS broth and incubated at 37 ^{o}C for 24 hrs in aerobic condition. The activated cultures were stored at 4 ^{o}C for use throughout the course of the study. The cultures were revived after

every 10 days. For each trial, 24 hrs old, fresh culture was used.

2(c) Collection of test pathogens

The selected pathogen for the study, were collected from Microbial Culture Collection Bank, Department of Microbiology and Microbial Technology, College of Biotechnology and Allied Sciences, A.A.I.D.U, Allahabad.

Maintenance of test pathogens

The selected pathogens were maintained on nutrient agar slants and activated by transfer into nutrient broth and incubated for 24 hrs. The organisms were stored at 4 °C for use throughout the course of the study. For each trial, 24 hrs old cultures was used.

Probiotic Characterization

For testing probiotic characteristics of indigenous *L.acidophilus* isolates following tests were performed.

Effect of bile salt on indigenous *Lactobacillus acidophilus* isolates

3.1 (a) Effect on cell growth

The effect of Bile salt (sodium taurocholate) was studied as per the procedure laid by Goderska and Czarnecki, (2007). MRS broth was prepared and 100-ml broth was distributed in 150 mL conical flasks. Bile salt was added to each flask and was adjusted up to 0.1% and 0.2% concentration. A control was maintained without bile salt. Ten mL broth from each conical flask was transferred in culture tubes and plugged properly with cotton. The medium was sterilized at 121 °C for 20 minutes. After cooling 1 mL of 24 hour grown inoculums of all the indigenous *L. acidophilus* isolate was added separately to all the treatment tubes and were kept for incubation at 37±0.5 °C for 1 hours, 3 hours and 6 hours. After incubation 1 mL of the sample from each tube was serially diluted in sterile peptone water and sensible dilution (10^7) was plated in triplicates on the selective medium by pour plate method and the plates were incubated at 37±0.5 °C for 24 hours under anaerobic condition. The number of colonies were counted and recorded as cfu/mL and also compared with the count of treatment control. The percentage reduction in cell count of *L.acidophilus* isolates were calculated with the help of formula given below.

$$\text{Percentage Reduction} = \frac{\text{Treatment cell count}}{\text{Control count}} \times 100$$

3.1(b) Effect on bacteriocin production

Test for bacteriocin production was done according to Aslim *et al.* (2005). Each *L. acidophilus* isolate was inoculated in 100 mL of MRS broth with different concentration of bile salt and then incubated anaerobically using candle jar extinction method at 37°C for 24 to 48 hrs. It was then be centrifuged at 8000 rpm for 15 min. The supernatant was collected and decanted into sterile test tubes, adjusted to pH 6.5-7.0 with NaOH (40G/1000 mL) to remove organic acid effect. The diluted supernatant of LAB culture were filtered and sterilized with 0.2 µm Millipore filter membrane. The titers of bacteriocin produced was quantified by two fold serial dilution of bacteriocin in 0.8% saline solution up to the sensible dilution (10^4). Inhibitory effect of free bacteriocin on test pathogen bacteria were then determined by agar well diffusion method. For this pre-prepared nutrient agar plates were used. The 24 hrs old broth culture of test pathogen was swabbed on nutrient agar plates and wells of 5mm diameter were cut in the plates. Now 0.1 mL supernatant from each dilution tube was transferred in respective well. The inoculated plates were incubated at 37 °C for 24 h along with treatment control, media control and organism control. The diameter of the inhibition zone was measured according to the methods outlined by Aslim *et al.* (2005). The antimicrobial activity of the bacteriocin was defined as reciprocal of the highest serial dilution showing inhibition of the indicator isolate and was expressed in activity units per ml (AU mL^{-1}) (Graciela *et al.*, 1995).

Effect of pH on indigenous *Lactobacillus acidophilus* isolates

3.2(a) Effect on cell growth

The effect of pH was performed by the procedure laid by Murthy *et al.*(2000). MRS broth was prepared and 100 mL broth was distributed in the 150 mL of conical flasks and different levels of pH were adjusted by using 1N HCl and 40% NaOH along with the control viz 2.0, 4.5, 6.5 and 8.0. Ten mL broth from each conical flask was transferred in culture tubes and plugged properly with cotton. The medium was sterilized at 121^0C for 20 minutes. After cooling 1 mL of 24 hrs grown culture inoculums of all the isolates were added separately to all the tubes and kept for 37±0.5^{0}C for 1hour, 3

hour and 6 hours incubation. After the incubation 1 mL of the sample from each treatment tube along with control were serially diluted in sterile peptone water and sensible dilution (10^7) was plated in triplicates on selective medium, *i.e.* MRS agar by pour plate method. All the plates were incubated at 37±0.5 °C under anaerobic condition for 24 hours. After proper incubation the number of colonies were counted and were expressed as cfu/mL. The percentage reduction in cell count of *L. acidophilus* isolates was calculated using earlier discussed formula.

3.2(b) Effect on bacteriocin production

Test for bacteriocin production was done as discussed earlier according to Aslim *et al.* (2005).

4. Statistical analysis

The obtained data during the course of investigation were analyzed statistically using analysis of variance and critical difference test (Fisher and Yates, 1953) and the results were interpreted.

RESULTS

Primary Screening of Lactobacillus acidophilus isolates

The antimicrobial activity of all the five isolates *Lactobacillus acidophilus* was screened against *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* and *Salmonella typhi* by agar well diffusion method. Isolate *Lactobacillus acidophilus* (LA1) showed maximum zone of inhibition against *Bacillus cereus* (13.00mm), followed by *Staphylococcus aureus* (11.00 mm), *Escherichia coli (*10.33 mm*)* and minimum zone of inhibition was observed in *Salmonella typhi* (10.33 mm). In case of *Lactobacillus acidophilus* (LA2) the maximum sensitivity was observed in *Staphylococcus aureus* as well as *Bacillus cereus* (12 mm), and least against *Salmonella typhi* (8.00 mm), however zone of inhibition showed by *Escherichia coli* was 9.67 mm. In case of *Lactobacillus acidophilus* (LA3) maximum zone of inhibition was found against *Staphylococcus aureus* (16.00 mm), slight decrease against *Bacillus cereus* (14.00 mm) and zone of inhibition against *Escherichia coli* was 10.33 mm. In case of *Lactobacillus acidophilus* (LA4) least inhibition zone was found against *Escherichia coli* and more against *Salmonella typhi* 12.00 and 14.33 mm respectively. Zone of inhibition against *Bacillus cereus* and *Staphylococcus aureus* was found to be 13.67 and 13.33 mm respectively. In case of LA5 Zone of inhibition was highest against *Staphylococcus aureus* and lowest against *Salmonella typhi*, 14.00 and 9.00 mm respectively. *Escherichia coli* showed 12.00 mm zone of inhibition while slight increase was observed against *Bacillus cereus* 12.33 mm. The results showed that maximum zone of inhibition was found against *Staphylococcus aureus* (16.00 mm) by *Lactobacillus acidophilus* (LA3) strain and minimum zone of inhibition was observed against *Salmonella typhi* (8.00 mm) by *Lactobacillus acidophilus* (LA2) isolate. *Escherichia coli* and *Bacillus cereus* show diffuse type of zone of inhibition in case of all strains. There are several reports that suggested that *Lactobacillus acidophilus* inhibits growth of various pathogenic and spoilage bacteria, because they produce various compounds such as organic acids, diacetyl , hydrogen peroxide and bacteriocin or bactericidal proteins during lactic

Table 1. Antimicrobial activity of *Lactobacillus acidophilus* isolates

No.of isolates	Average diameter of zone of inhibition (mm)			
	Staphylococcus aureus	*Bacillus cereus*	*Escherichia coli*	*Salmonella typhi*
LA1	11.00	13.00	10.33	9.00
LA2	12.00	12.00	9.67	8.00
LA3	16.00	14.00	13.33	10.33
LA4	13.33	13.67	12.00	14.33
LA5	14.00	12.33	12.00	9.00

Zone size includes 5mm diameter of well

S (due to pathogens); S.E=0.88, C.D (5%) =1.91 after calculating C.D following combinations were found to be significant; (*Staphylococcus aureus Salmonella typhi*), (*Bacillus cereus, Salmonella typhi*), where (*Staphylococcus aureus, Escherichia coli*), (*Staphylococcus aureus, Bacillus cereus*), (*Bacillus cereus, Escherichia coli*) and (*Escherichia coli, Salmonella typhi*) were nonsignificant.

S (due to isolates): S.E=0.79, C.D (5%) =1.72 after calculating C.D following combination were found to be significant. (LA3, LA2), (LA3, LA1), (LA4, LA2), (LA4, LA1) where (LA3, LA5) (LA4, LA5), (LA5, LA2), (LA1,LA2) were found to be nonsignificant

fermentations (Ogunbanwo *et al.,* 2003; Goderska and Czarnecki, 2007).

From the ANOVA Table it is evident that the calculated values of F due to organism and due to isolates are greater than the Table values of at 5% probability level and on their respective degree of freedom. So there is significant effect of isolates as well as of organisms on the antagonistic activity of *Lactobacillus acidophilus.*

Effect of bile salt on cell growth

In the present study, effect of different bile salt concentration on the growth of *L.acidophilus* isolates were studied. The strains were tested for their bile tolerance capacity at different concentration of bile salt (0.1% and 0.2%). The time intervals used for incubation were 1 hour, 3 hours and 6 hours.The LA-1 isolate was treated with both the bile salt concentration for 1 hour, 3 hours and 6 hours incubation and an increase in percentage reduction was observed with respect to increase concentration of bile salt, *i.e.* 0.1%-48.1% and 0.2%- 52.4% for 1 hour incubation, for 0.1%-54.4% and 0.2%- 58.48% at 3 hours incubation time and for 6 hours it was found to be 0.1%- 57.28% and 0.2%- 61.22%, respectively; in comparison to control where no bile salt was present. When LA-2 isolate was treated with different bile salt concentrations, the percentage reduction at 1 hour was observed as 38.7% and 52.61% for 0.1% and 0.2% bile salt concentration, for 3 hours incubation percentage reduction was 0.1%-55 % and 0.2%- 63.77% and for 6 hours the percentage reduction was 55.82% and 64% for 0.1% and 0.2%, respectively, in comparison to control.In case of LA-3 isolate for 1 hour, 3 hours and 6 hours incubation at different bile salt concentration (0.1% and 0.2%) the reduction in cell count was found to be 2.80%, 22.19%, 29.74%, 46.18% and 35.39%, 50.33%, respectively. For LA-4 isolate the percentage reduction at the 1 hour incubation was found to be 0.1%- 34.46%, 0.2%- 38.35%, for 3 hours incubation, 0.1%-38.96%, 0.2%- 42.84% , and 6 hours incubation the percentage reduction in cell count was 48.93% and 52.09% respectively. In the case of LA-5 isolate of *L.acidophilus* for 1 hour incubation, the percentage reduction was found to be 0.1%-40.27%, 56.43%, for 3 hours incubation time, 0.1%-43.24% and 0.2%- 48.62% and 6 hours incubation the percentage reduction was found 47.39% and 57.29% respectively. On comparing the percentage reduction of all five *L. acidophilus* isolates at different time intervals and bile salt concentration, it was observed that LA-3 was found to be most bile tolerant isolate at 0.1% concentration and at different time interval. Again on 0.2% bile salt, maximum tolerance was observed with LA-3 at 1 and 6 hours incubation. However at this concentration LA-4 was showing maximum tolerance after 3 hours of incubation.

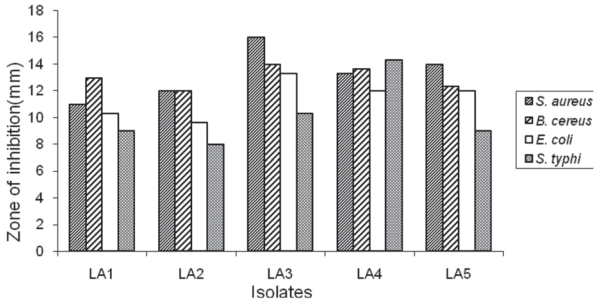

Fig. 1. Antimicrobial activity of *Lactobacillus acidophilus* isolate

Effect of bile salt on antimicrobial activity of *Lactobacillus acidophilus* isolates

In the present study, the effect of different concentration of bile salt on antimicrobial activity of

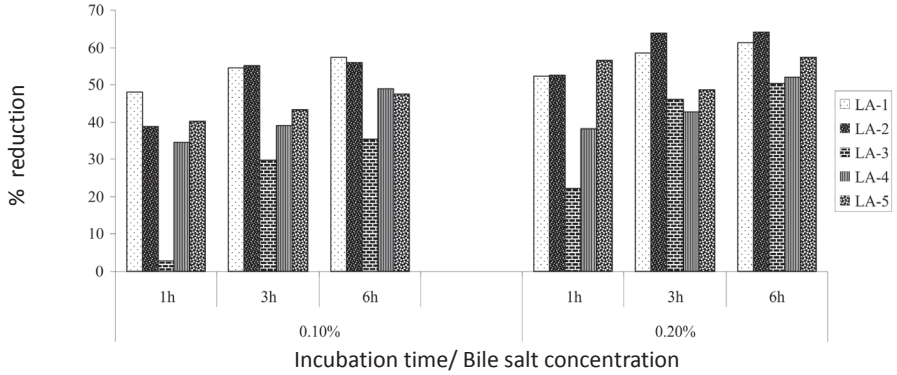

Fig. 2. Comparative growth of *Lactobacillus acidophilus* at different concentration of bile salt and time intervals.

L.acidophilus was studied. The effect of bile salt on antimicrobial activity was evaluated for five *L. acidophilus* isolates, against common food spoilage and enteric pathogenic bacteria *i.e. S. aureus, B. cereus, E.coli* and *S.typhi.* On the basis of size of zone of inhibition, the antimicrobial activity of isolates was interpreted. From the study, it was observed that when LA-1 was treated at different bile salt concentration, maximum antimicrobial activity was observed against *E.coli* at 0.1% (15 mm) bile salt concentration followed by 0.2% (12 mm). For *S.aureus,* maximum activity was observed at 0.1% (15 mm) and minimum activity was observed at 0.2% (13 mm). For *B. cereus* moderate activity at different concentration of bile salt, *i.e.* 0.1% (13 mm) and 0.2% (11 mm) was observed. The *S.typhi*, was least inhibited at both concentration of bile salt, *i.e.* 0.1% (12 mm) and 0.2% (10 mm). When LA-2 isolate was treated with same bile salt concentrations, the maximum antimicrobial activity against *S. aureus* was observed at 0.1% (14 mm) and 0.2%, 12 mm zone of inhibition. For *B.cereus* moderate antimicrobial activity was observed, *i.e.* 0.1% (12 mm) and 0.2% (10 mm). The *E. coli* and *S. typhi*, was same inhibited at both concentration of bile salt, *i.e.* 0.1% (13 mm) and 0.2% (11 mm). LA-3 isolate was treated with different concentration of bile salt, the maximum activity was observed against *S.aureus*, at 0.1% (16 mm) and 0.2% (13 mm). The *B. cereus* showed moderate zone of inhibition at both concentration of bile salt *i.e.* 0.1% (13 mm) and 0.2% (12 mm). *E.coli* showed maximum inhibition at 0.1% concentration of bile salt (15 mm) and minimum inhibition was observed at 0.2% concentration of bile salt (13 mm). The *S.typhi* showed least inhibition at both concentration of bile salt, *i.e.* 0.1% (13 mm) and 0.2% (11 mm). When LA-4 was treated with different bile salt concentration, moderate antimicrobial activity was observed, against *S. aureus* and *E. coli, i.e.* 0.1% (13 mm) followed by 0.2%, (11 mm). For *B. cereus* same antimicrobial activity was observed at

Table 2. Comparative growth of *Lactobacillus acidophilus* at different concentration of bile salt and time intervals:

Bile salt conc.(%)	Time period	LA-1		LA-2		LA-3		LA-4		LA-5	
		cfu/ml	% reduction	cfu/ml	% reduction	cfu/ml	% reduction	cfu/ml	% reduction	cfu/ml	% reduction
0.1%	1h	21.46	48.1	19.34	38.7	30.7	2.80	22.6	34.46	22.76	40.27
	3h	22.4	54.4	20.53	55.0	33.55	29.74	23.6	38.96	24.6	43.24
	6h	24.5	57.28	24.3	55.82	35.25	35.39	5.26	48.93	29.57	47.39
0.2%	1h	19.7	52.4	14.93	52.61	24.2	22.19	20.63	38.35	21.5	56.43
	3h	20.36	58.48	16.51	63.77	25.7	46.18	22.1	42.84	22.27	48.62
	6h	22.2	61.22	9.8	64.0	27.1	50.33	23.7	52.09	23.7	57.29
Control	1h	41.34		31.5		31.1		33.46		38.1	
count	3h	49.03		45.53		47.75		38.66		43.34	
	6h	57.34		55.0		54.55		49.46		56.2	

Significant due to incubation time: SE= 2.63, CD (5%)= 5.44
Incubation time= 6h 3h 1h
Mean= 105.94 > 96.24 > 77.26

Significant due to bile salt conc: SE= 2.15, CD (5 %) = 4.45
Bile salt conc= 0.1% 0.2%
Mean= 153.36 > 126.09

Significant due to different isolates: SE= 3.40, CD (5%) = 7.03
Isolates = LA1 LA2 LA5 LA4 LA3
Mean = 55.31>54.98>48.87>42.6 >31.10

0.1% concentration of bile salt (13 mm) and least activity was observed at 0.2% (10 mm). The *S.typhi* showed least inhibition at both concentrations of bile salt, *i.e*. 0.1% (12 mm) and 0.2% (11 mm). In the case of LA-5 isolate, moderate antimicrobial activity against different pathogenic organisms at different concentration of bile salt was observed. For *E.coli,* maximum activity was observed at 0.1% (14 mm) and minimum activity at 0.2% (13 mm). For *S. aureus*, maximum activity was found at 0.1% (13 mm) and minimum activity at 0.2% (12 mm) concentration of bile salt. *B. cereus* and *S. typhi* showed same inhibition at 0.1% concentration of bile salt, *i.e*. 12 mm and followed by 0.2% concentration of bile salt, *i.e.* 11mm and 10 mm.

In this study it was found that all the five *L.acidophilus* isolates treated with different concentration of bile salt were showing inhibition in the growth of test pathogens. But out of five *L.acidophilus* isolates, LA-1 isolate was found to be moderatelyeffective against *E.coli* and *S.aureus* at both concentrations. However, LA-3 isolate was found to be most effective against all test pathogens. It was statistically analyzed that the antimicrobial activity was found to be significantly different with respect to *L. acidophilus* isolates and different concentration of bile salt, and showed significant difference with respect to different test pathogens. After calculating the CD value following combination were found to be significant- (*S. aureus, E.coli*), (*E. coli, B.cereus*), (*S.aureus, B. cereus*) and (*S.aureus, S.typhi*) whereas (*B.cereus, S.typhi*) was found to be non significant. For different isolates, after calculating CD value following combination were found to be significant (LA3, LA1), (LA3, LA5), (LA3, LA2), (LA3, LA4), (LA1, LA5) and (LA1, LA2) whereas (LA5, LA2) and (LA2, LA4) were found to be non significant. For different concentration of bile salt after calculating the CD value (0.1%, 0.2%) was found to be significant.On the basis of above data, LA-3 isolates can be considered as the best antagonistic organisms among the five isolates. The activity unit of the bacteriocin produced by *L.acidophilus* isolates at different bile salt concentration was found to be 400 AU/mL.

Effect of pH on Cell Growth

In the present study, effect of different pH on the growth of *L. acidophilus* was studied. The isolates were tested for their acid tolerance capacity at different pH level and for different time intervals. From the study it was found that *L.acidophilus* isolates (LA-1, LA-2, LA-3, LA-4, LA-5), grown at different pH levels *i.e*. 2.0, 4.5, 8.0 and 6.5 (control), showed remarkable reduction in cell count at each pH levels for different time Interval, *i.e.* 1 hours, 3 hours, and 6 hours. When LA-1 isolate was subjected to different pH level for 1 hour, 3 hours, and 6 hours incubation showed a gradual increase in percentage reduction with respect to decrease in pH. Percentage reduction observed for 1 hour

Table 3. Effect of bile salt on antimicrobial activity of *Lactobacillus acidophilus* isolates against different pathogens:

Pathogens	*Lactobacillus acidophilus* isolates									
	LA-1		LA-2		LA-3		LA-4		LA-5	
	Zone of inhibition at different bile salt concentration (mm)									
	0.1%	0.2%	0.1%	0.2%	0.1%	0.2%	0.1%	0.2%	0.1%	0.2%
S. aureus	15.0	13.0	14.0	12.0	16.0	13.0	13.0	11.0	13.0	12.0
B. cereus	13.0	11.0	12.0	10.0	13.0	12.0	13.0	10.0	12.0	11.0
E. coli	15.0	12.0	13.0	11.0	15.0	13.0	13.0	11.0	14.0	13.0
S. typhi	12.0	10.0	13.0	11.0	13.0	11.0	12.0	11.0	12.0	10.0

Significant due to bile salt concentration: SE= 0.20, CD(5%)=0.40,
Bile salt concentration= 0.1% 0.2%
Mean= 53.2 > 45.6
Significant due to pathogens: SE= 0.28, CD(5%)= 0.56,
Pathogens= S.aureus E.coli B.cereus S.typhi
Mean= 13.2 > 13.0 > 11.7 > 11.5

Significant due to isolates: SE= 0.32, CD(5%)= 0.64,
Isolates= LA3 LA1 LA5 LA2 LA4
Mean= 26.5 >25.25>24.25 >24 >23.5

incubation (pH 2-27.2%, pH 4.5-21.4% and pH8-26.47%) 3 hours incubation (pH 2-42.02%, pH 4.5-26.1%, pH 8-29.58%) and for 6 hours incubation (pH2- 59.14%, pH4.5-13.92%, pH8-36.47%) respectively, was higher in comparison to control, *i.e.* pH 6.5. For LA-2, percentage reduction observed for different pH for 1 hour (pH2- 17.85%, pH4.5-11.6%, pH8-53.72%), for 3 hours (pH2-50.4%, pH-4.5- 17.6%, pH8-43.77%) and for 6 hour incubation (pH2- 64.44%, pH4.5- 14.49%, pH8- 41.98%) in comparison to pH 6.5 as control. When LA-3 isolate was treated with same pH level the percentage reduction for 1 hour incubation (pH2- 9.2%, pH4.5-1.10%, pH8- 7.0%) For 3 hour (pH2- 41.8%, pH4.5-20.2%, pH8-31.2%) and for 6 hour incubation (pH2-57.28%, pH4.5- 22.6%, pH8- 29.6%) with comparison to control For LA-4 (pH2-16.6%, pH4.5- 6.60%, pH8-14.14%) at 1 hour, (pH2- 32.49%, pH4.5 - 11.44%, pH8- 20.4%) at 3 hour, (pH2- 56.88%, pH4.5 - 16.66%, pH8- 34.44%) at 6 hour incubation time with comparison to control. For LA-5, reduction was observed at 1 hour, pH2- 25.31%, pH4.5- 17.75%, and pH8- 20.7%, for 3 hour, pH2- 41.63%, pH4.5-14.35%, pH8- 20.78%) and 6 hour incubation time pH2- 63.6%, pH4.5- 24.81%, pH8- 29.95%) in respect to control. From the above data, it was found that all the five isolates of *L.acidophilus* were showing remarkable gradual increase in percentage reduction with respect to pH. However out of five isolates, LA-3 isolate was found to be most acid tolerant at pH2, for 1 hour incubation, at the same pH LA-4 showed minimum percentage reduction at 3 hour incubation period, and at same pH LA-2 showed maximum percentage reduction at 6 hour incubation. Minimum percentage reduction was shown by LA-4 at 6 hour incubation. Again when *L.acidophilus* isolates were screened at pH 4.5, LA-3 isolate was showing minimum percentage reduction for 1 hour incubation, at same pH LA-4 isolate was showing least reduction at 3 hour incubation and at same pH 4.5 and LA-1 showed minimum percentage reduction at 6 hour time intervals. At pH8, LA-3 isolate was found to be most tolerant for 1 hour incubation, i.e. 7.0 %. LA-4 showing maximum acid tolerant for 3 hour, *i.e*. 20.4 % and LA-3 was showing most acid tolerant at 6 hour incubation, *i.e.* 29.6 %. All the five *L. acidophilus* isolates showed slight variation in their percentage reduction in colony count at different pH levels and three different time intervals. Statistically, there was non-significant difference in percentage reduction in cell count with respect to five different isolates. However, different time interval and different pH were significant. After calculating the CD for time intervals all combination were found to be significant, *i.e.* (1h, 3h), (1h, 6h), and (3h, 6h). For different pH, all combination were found to be significant, *i.e*. (pH6.5, pH4.5), (pH6.5, pH8), (pH6.5, pH2), (pH4.5, pH8), (pH4.5, pH2) and (pH8, pH2). Therefore on the basis of acid tolerance, LA-3 isolate was found to be most acid tolerant isolate among the all five isolates.

Effect of pH on antimicrobial activity of *Lactobacillus acidophilus* isolates

In the present study, the effect of different pH on the antimicrobial activity of *L.acidophilus* was studied. The effect of pH on the antimicrobial activity was

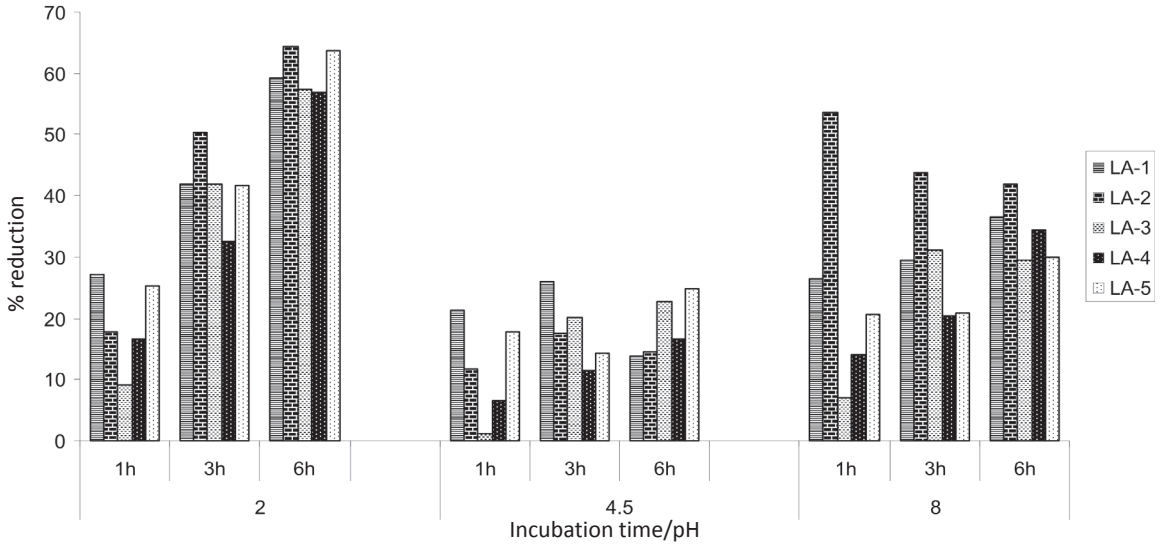

Fig. 3. Comparative growth of *Lactobacillus acidophilus* isolates at different pH and time intervals.

evaluated for five *L.acidophilus* isolates against common food spoilage and enteric pathogenic bacteria, *i.e. S.aureus, B.cereus*, *E.coli* and *S.typhi.* Onthe basis of the size of the zone of inhibition, the antimicrobial activity of isolates was interpreted. From the study, it was observed that when LA-1 was treated at different pH levels, maximum activity against *S. aureus* was observed in pH 6.5 (13.0 mm) followed by pH4.5 (12.0 mm), pH 8 (11.0 mm) and least activity was observed in pH2 (10.0 mm). The maximum antimicrobial activity for *B.cereus* was observed at pH 6.5 (13.0 mm) followed by pH4.5 (11.0 mm), pH2 (10.0 mm) and least activity was observed in pH8 (9.0 mm). The *E.coli* was showing maximum activity at 6.5 (14.0 mm), followed by 4.5 (13.0 mm), pH8 (10.0 mm) and least activity was observed at pH2 (9.0 mm). In the case of *S.typhi* , minimum activity at different pH, *i.e.* pH6.5 (12.0 mm), pH4.5 (11.0 mm), pH8 (9.0 mm) and least activity at pH2 (8.0 mm) was observed. When LA-2 isolate was treated with same pH level, the maximum antimicrobial activity against *S.aureus* was observed at pH6.5 (15.0 mm), followed by pH4.5 (13.0 mm), and pH8 (11.0 mm) and minimum activity was found to be at pH2 (10.0 mm). For *B.cereus* at pH 6.5 and 4.5 the isolate showed same activity (13.0 mm) followed by pH8 (12.0 mm) and at pH2 least activity was observed (11.0 mm). For *E.coli*, maximum activity was detected at pH 6.5 (14.0 mm), at pH 4.5 and 8 same activity (12.0 mm) was found and at pH2 (10.0 mm) least activity was reported. For *S.typhi*, least activity was observed at different pH in comparison to other pathogens. It was observed (at 6.5 pH) 12.0 mm, followed by (4.5 pH) 11.0 mm, (8 pH) 10.0 mm and (2 pH) 9.0 mm. When LA-3, was treated with different pH, moderate activity was observed against *S.aureus, i.e.* in pH6.5 (16.0 mm), 4.5 (13.0 mm), 8.0 (10.0 mm) and 2.0 (11.0 mm). Again *B. cereus,* showed moderate zone of inhibition at pH 6.5 (14.0 mm), followed by pH4.5 (12.0 mm), then pH8 (10.0 mm) and minimum activity was seen at pH2 (10.0 mm). Among the gram-negative bacteria, *i.e. E.coli* and *S.typhi*, maximum inhibition was observed in the case of *E.coli* at different pH levels, *i.e.* at pH6.5 maximum activity was observed with 12.0 mm zone of

Table 4. Comparative growth of *Lactobacillus acidophilus* isolates at different pH and time intervals:

pH	Incubation time	LA-1		LA-2		LA-3		LA-4		LA-5	
		cfu/ml	% reduction	cfu/ml	% reduction	cfu/ml	% reduction	cfu/ml	% reduction	cfu/ml	% reduction
2	1h	30.10	27.2	25.55	17.85	28.63	9.2	27.93	16.6	28.46	25.31
	3h	28.43	42.02	23.7	50.46	26.5	41.8	26.1	32.49	25.3	41.63
	6h	23.43	59.14	19.4	4.44	23.5	57.28	21.33	56.88	20.46	63.6
4.5	1h	32.53	21.4	27.5	11.6	31.16	1.10	31.2	6.60	31.34	17.75
	3h	36.26	26.1	39.35	17.6	36.36	20.2	34.24	11.44	37.16	14.35
	6h	49.36	13.92	46.65	14.49	42.6	22.6	41.47	16.66	42.26	24.81
8	1h	30.4	26.47	25.25	53.72	29.3	7.0	28.73	14.14	30.24	20.7
	3h	34.53	29.58	26.85	43.77	31.36	31.2	30.63	20.4	34.34	20.78
	6h	36.43	36.47	31.65	41.98	38.76	29.6	32.43	34.44	39.37	29.95
6.5 (C)	1h	41.34		31.1		31.5		33.46		38.1	
	3h	49.03		47.75		45.53		38.66		43.34	
	6h	57.34		54.55		55.0		49.46		56.2	

Significant due to incubation time: SE= 3.34, CD (5%)= 6.71
Incubation time= 6h 3h 1h
Mean= 168.61>140.54>101.47

Significant due to different pH :SE= 3.86, CD (5 %)= 7.75
pH= 6.5pH 2pH 8pH 4.5pH
Mean = 134.44>115.04>98.56>62.56

inhibition, followed by pH4.5 (11.0 mm) however at pH2 and pH8 same zone size (activity) of 9.0 mm was observed *S.typhi* was less inhibited by LA-3 in comparision to *E.coli*. For *S.typhi,* the zone of inhibition different at pH, *i.e*. 6.5, 4.5, 8.0, and 2.0 was observed as 13.0 mm, 13.0 mm, 12.0 mm and 11.0 mm respectively. When LA-4 isolate was treated at different pH levels, maximum activity against *S. aureus* was observed at pH 6.5 (12.0 mm) followed by pH4.5 (10.0 mm), pH2 (11.0 mm) and least activity was observed in pH8 (10.0 mm). The maximum zone of inhibition against *B.cereus*, was found to be same at pH6.5 and 4.5 (11.0 mm), at pH2 the zone was 9.0 mm and minimum zone of inhibition was showed at pH8 (8.0 mm). For *E.coli* maximum activity was observed at pH6.5 (13.0 mm), the same activity showed at pH4.5 and pH2 (12.0 mm) and minimum activity at pH8 (11.0 mm). And *S.typhi* showed least antimicrobial activity, at the different pH followed by in pH6.5 (12.0 mm), pH4.5 (10.0 mm), pH2 (9.0 mm) and no activity was showed at pH8.LA-5, showed moderate antimicrobial activity against different pathogenic bacteria at different pH levels. The *S. aureus* showed same activity at 6.5 and 4.5 pH *i.e*. 13.0 mm, and at pH8 showed (11.0 mm) and minimum activity was observed at pH2 (10.0 mm). For *B. cereus*, moderate activity was observed at pH6.5 (12.0 mm) and pH4.5 (11.0 mm), however the organism showed same activity at 2 and 8 pH (9.0 mm). *E.coli* was showing maximum inhibition in pH6.5 (15.0 mm), followed by pH4.5 (13.0 mm), pH8 (12.0 mm) and minimum inhibition was observed at pH2 (11.0 mm). For *S.typhi* least activity was observed at different pH as pH6.5 (12.0 mm), pH4.5 (11.0 mm), pH8 (10.0 mm) and no activity was observed at pH2. It was statistically analyzed that the antimicrobial activity was found to be significantly different with respect to different *L. acidophilus* isolates, after calculating the CD value following combination were found to be significant: (LA1, LA2), (LA1, LA3), (LA1, LA4), (LA2, LA4), (LA2, LA5), (LA3, LA4), (LA3, LA5) and (LA4, LA5) whereas, (LA2, LA3) and (LA1, LA5) were found to be non-significant. And for different pathogens, after calculating the CD following combination were found to be significant: (*E.coli*, *S.typhi*), (*S. aureus, S. typhi*) and (*B.cereus, S.typhi*) whereas, (*S.aureus, E.coli*), (*B.cereus, E.coli*) and (*S.aureus, B.cereus*) were

Table 5. Effect of pH on antimicrobial activity of *Lactobacillus acidophilus* isolates against different pathogens:

Pathogens	*Lactobacillus acidophilus* isolates																			
	LA-1			LA-2			LA-3			LA-4			LA-5							
	Zone of inhibition at different pH (mm)																			
2	4.5	6.5	8	2	4.5	6.5	8	2	4.5	6.5	8	2	4.5	6.5	8	2	4.5	6.5	8	
S. aureus	10.0	12.0	13.0	11.0	10.0	13.0	15.0	11.0	11.0	13.0	16.0	10.0	9.0	10.0	12.0	10.0	10.0	13.0	13.0	11.0
B.cereus	10.0	11.0	13.0	9.0	11.0	13.0	13.0	12.0	10.0	12.0	14.0	10.0	9.0	11.0	11.0	8.0	9.0	11.0	12.0	9.0
E.coli	9.0	13.0	14.0	10.0	10.0	12.0	14.0	12.0	9.0	11.0	12.0	9.0	12.0	12.0	13.0	11.0	11.0	13.0	15.0	12.0
S.typhi	8.0	11.0	12.0	9.0	9.0	11.0	12.0	10.0	11.0	13.0	13.0	12.0	9.0	10.0	12.0	-	-	11.0	12.0	10.0

Significant due to different pH: SE= 0.54, CD(5%)= 1.08
pH=6.5pH 4.5pH 8pH 2pH
Mean=65.25 > 59 > 49 > 46.75

Significant due to different pathogens: SE= 0.54, CD(5 %)= 1.08
Pathogens=E.coli S.aureus B.cereus S.typhi
Mean= 11.7 >1.65 >10.9 > 9.75

Significant due to isolates: SE= 0.61, CD(5%)= 1.21
Isolates= LA2 LA3 LA1 LA5 LA4
Mean= 47 > 46.5 >43.75> 43 > 39.75

found to be non-significant. For different pH treatment, all combination were found to be significant *i.e.* (pH6.5, pH4.5), (pH6.5, pH8), (pH6.5, pH2), (pH4.5, pH8), (pH4.5, pH2) and (pH8, pH2). Therefore, on the basis of above data, LA-3 isolates can be considered as the best antagonistic organism among the five *L. acidophilus* isolates. The activity unit of the bacteriocin produced by *L.acidophilus* isolates at different pH level was found to be 400 AU/mL.

DISCUSSION

Effect of bile salt on cell growth

All the five isolates of *L.acidophilus* were showing variable percentage reduction at different bile salt concentration for 1 hour, 3 hours and 6 hours incubation. The percentage reduction with respect to bile salt concentration and time intervals was found to be significant. After calculating the CD for bile salt concentration following combination was found to be significant: (0.1%, 0.2%). For different time intervals all combination were found to be significant, *i.e.* (1h, 3h), (1h, 6h) and (3h, 6h). And also for the different isolates of *L.acidophilus*, the difference was significant. After calculating the CD following combinations were found to be significant: (LA1, LA3), (LA1, LA4), (LA2, LA3), (LA2, LA4), (LA3, LA5) and (LA3, LA4). Whereas, (LA1, LA2), (LA1, LA5), (LA2, LA5) and (LA4, LA5) were found to be non-significant. (Table 4.1, Fig 4.1, Plate no. 4.1(a) and 4.1(b). Therefore on the basis of bile tolerance LA-3 isolate can be considered as most bile tolerant organism among the five isolates. Almost same types of findings were reported by various authors (Murthy *et al.,* 2000; Goderska and Czarnecki, 2007; Rashid *et al.,* 2007; Tsuda *et al.,* 2007; Ashraf *et al.,* 2009). Gastrointestinal systems have varying concentrations of bile. The rate of secretion of bile acid and its concentration depend on the type of food consumed. For example, the fat content of the diet could influence the level of faecal bile acids and fatty acids may increase the inhibitory effects of bile acids towards *Lactobacillus spp*. Bile concentrations range from 0.5 to 2.0% in the first hour of digestion and the levels may decrease during the second hour (Kailasapathy and Chin, 2000). They reported that gradual decrease in cell count with increase in bile salt concentrations could be due to difference in bile salt resistence and natural difference in growth of individual isolates. A concentration of 0.05 to 0.1% of bile salt is secreted into the intestine. Thus for any bacterium to survive in the stomach and proliferate in the intestine, it should tolerate bile salt (Murthy *et al.,* 2000). Free bile acids adhere to bacteria or dietary fiber, thus enhancing excretion of bile acids. This action may trigger the feedback mechanism that regulates hepatic cholesterol synthesis and subsequent transformation into bile acids, which may reduce serum cholesterol concentrations (Chikai *et al.,* 1992). Lankaputhra and Shah (1995) reported that among six strains of *Lactobacilli,* two strain of *L.acidophilus* showed the best tolerance of bile (1-1.5%). Floch *et al.* (1972) reported that deconjugation bile acids were more inhibitory to microorganisms than conjugated bile acids and suggested that this inhibitory effect may play a role in inhibiting microbial growth in the intestinal tract. It was most likely not a detoxification mechanism for these organisms (McAuliffe *et al.,* 2005). *Lactobacillus acidophilus*, which was the least bile-resistant strain tested, decojugated approximately twice as much sodium taurocholate during 3 h of incubation. Dashkeviez and Feighner (1989) in their assays reported that the upper concentration of bile salts used was reduced from 0.5% to 0.2% due to the inability of *L. acidophilus* NCFM to grow at 0.5%. Bile salt is detergent and disorganize the membrane lipid structure, and it is difficult for microorganisms to live under high concentration of bile salts conditions. Although the native gastrointestinal microorganisms must have developed strategies to defend themselves against the toxic action of bile salts, the resistant mechanisms of these bacteria are poorly understood (Tsuda *et al.,* 2007). The 2% bile salts used for testing our strains represents the extreme concentration obtained in animal or human intestine during the first hour of digestion (Gotcheva *et al.,* 2002). After wards the normal level of bile salt in intestine is around 0.3% (Xanthopoulos *et al.,* 1997). At 0.1% bile salt concentration, more than 80% of *L.acidophilus* DDS-1 survived in comparison to the 73, 58 and 42% viability in the case of *L.acidophilus* strains NRRL 4495, NRRL 629, NRRL 1910. During exposure to increasing bile salt concentrations, the viability of all cultures decrease gradually, with a total loss at 0.25% bile salt concentrations (Murthy *et al.,* 2000). In the present study LA3 was found to be most bile tolerant strains, which could be due to the fact thatthe difficulty in lysing the *L.acidophilus* ADH suggests the presence of a much more resistant cell wall (Conway *et al.,* 1996).

Effect of bile salt on antimicrobial activity of *Lactobacillus acidophilus* isolates

There were several reports that suggest *L. acidophilus* inhibit the growth of various enteric pathogenic and spoilage bacteria at different bile salt concentrations (Shahani *et al.,* 1979; Zamfir *et al.,* 2000; Goderska and Czarnecki, 2007). Antimicrobial activity as well as growth decrease in the presence of high concentrations of bile salts. Sodium glycocholate was known to be more inhibitory than sodium taurocholate at equimolar levels (Shahani *et al.,* 1979). The significant strain by broth interaction for antimicrobial activity and pH indicates that the *L.acidophilus* strain and nutrient medium are mutually dependent on each other for the growth inhibition of organisms. The authors also observed differences in antimicrobial activity in 12 different strains of *L.acidophilus* against *B.subtilis.* Additionally, when one strain of *L.acidophilus* was cultured in 14 different nutrient media, they observed that the growth medium was also important for growth inhibition. Incorporation of bile salts into the MRS broth caused a growth inhibition and consequently a reduction in the antimicrobial activity. The acidity and antimicrobial activity are inversely related. The higher pH and lower antimicrobial activity was due to the growth limitation of *L.acidophilus* in presence of bile salts Shahani *et al.* (1979). The antimicrobial activity of *L.acidophilus* was reduced in vitro in the presence of bile salts. This does not necessarily mean that the situation is similar in vivo as established by Floch *et al.* (1972). Some bacteriocins produced by gram-positive bacteria have a broad spectrum of activity. However, it was generally observed that bacteriocin from producer organism had no inhibitory effect on the organisms producing it (Sanni *et al.,* 1999).

Effect of pH on cell growth

There were several reports suggesting that *Lactobacillus acidophilus* was stable under both acid and alkaline conditions (Murthy *et al.,* 2000; Goderska and Czarnecki, 2007; Rashid *et al.,* 2007; Tsuda *et al.,* 2007; Ashraf *et al.,* 2009). Bacteria would contact pH values ranging from 2.0 to 8.0 in the gastrointestinal tract if consumed (Rashid *et al.,* 2007). Due to the secretion of gastric juice, stomach normally has a pH of approximately 2.0. The highly acidic pH act as a defense mechanism by killing pathogenic microorganisms entering the body with the food we eat. The pH in the small intestine is generally 8.0 due to the secretion of pancreatic juices. Thus for any bacterium to survive in the stomach and proliferate in the intestine, it should be stable at low and high pH levels (Murthy *et al.,* 2000). *L.acidophilus* observed that the capability to survive in different pH of the environment is also a characteristic feature for bacterial strains. All the tested bacterial strains of *L.acidophilus* are characterized by significant longer survivability in the environment of pH characteristic for the individual segments of the gastrointestinal tract (Goderska and Czarnecki, 2007). In the case of the oral cavity, pH ranges from 6.2 to 7.4, depending on the speed of saliva secretion (Keller, 2000). Similar pH conditions prevail also in the bowels in which the undigested parts of food reach the large intestine within the period of 8 to 9 h (Ganong, 1994). It is true that bacteria are exposed to low pH of 2 to 3 in the stomach but it should be remembered that the environment of this part of the gastrointestinal tract is buffered by food. Although the time the food remains in the stomach is maximally 4 hours, bacteria are exposed to low pH for only several dozen minutes (Ganong, 1994). Low pH environments inhibit the metabolic activity and growth of *L.acidophilus* which are harmful to the bacterial cells, reducing their viability. The results of Bolin *et al.* (1997) experiments indicated that the studied strains showed different survival abilities in the pH ranging from 1.5 to 6.5. According to the analysis of variables affecting bacterial survival during the passage through a stomach model, results of Pinto *et al.* (2006) showed that it is necessary not only to test the tolerance to low pH, but the action of enzymes like pepsine and lysozyme. Survival of *L.acidophilus* depend on the pH of the environments; low pH decrease their survival. Under acidic conditions (both *in vitro* and *in vivo*), probiotic oeganisms, such as *L.acidophilus* survive better than the traditional culture organisms (*L.delbrueckii* and *S.thermohpilus*) in yoghurt (Shah andJalen, 1990). Hood and Zottola (1998) reported that *L.acidophilus* shows a rapid decline in numbers at pH 2.0, but at pH 4.0 the number of viable cells do not decrease significantly. These results have been confirmed by Lankaputhra and Shah (1995), where they concluded that six strains of *L.acidophilus* studied survived well at pH 3.0 or above and the viable counts was $>10^7$ CFU/g after 3 h incubation. However, Playne (1993) has reported that *L.acidophilus* does not grow well below pH 4.0. Ruis *et al.* (1994) have reported that *L.acidophilus* has high cytoplasmic buffering capacity (pH 3.72-7.74).

Which may allow it to resist changes in cytoplasmic pH and gain stability under acidic conditions. *L.acidophilus* is more tolerant to acidic conditions. The reduction of organisms in lower concentrations of pH may be due to the increasing in H^+ concentrations in medium which involves enzyme inactivation or disruption of cell (Tortora *et al*., 2004).

Effect of pH on antimicrobial activity of *Lactobacillus acidophilus* isolates

There were several reports that suggests *L.acidophilus* inhibit the growth of various enteric pathogenic and spoilage bacteria at different pH levels (Zamfir *et al*., 2000; Oliveira *et al*., 2008; Karthikeyan and Santosh, 2009; Dobreva-Yosifova *et al*., 2009). *Lactobacillus acidophilus* exert antagonistic effect on the growth of pathogens such as *Staphylococcus aureus, Salmonella typhimurium, Yersinia enterocolitica* and *Clostridium perfringens* (Ozbas and Aytac, 1995). Probiotic bacteria increases resistance against intestinal pathogens through antimicrobial mechanisms. These include competitive colonization and production of organic acids, such as lactic and acetic acids, bacteriocins and other primary metabolities, such as hydrogen peroxide, carbon dioxide, and diacetyl (Mishra and Lanbert, 1996). By competitive colonization and production bacteria inhibit the adhesion of gastrointestinal pathogens to the intestinal mucosa (Conway *et al*., 1996). Production of organic acids, such as lactic and acetic acids, by probiotic bacteria lower intestinal pH and thereby inhibits the growth of pathogens. These organic acids also increase peristalsis, through which they indirectly remove pathogens by accelerating their rate of transit through the intestine (Laroia and Martin, 1990). Corbon dioxide and diacetyl synthesized by lactic acid bacteria inhibit growth of pathogens. Bacteriocins produced by *L.acidophilus* are active against a wide range of food-borne pathogens, depending on their specificity (Mishra and Lanbert, 1996). Messens and De vuyst (2002) reported that many LAB and bacteriocins display greater antibacterial activity at lower pH values (pH5 and below) and significant activity was observed up to pH9. Most of the Gram positive bacteriocins acts upon the cytoplasmic membrane and increasesits permeability. They often show a much broader spectrum of bactericidal activity (Jack *et al*., 1996). Apart from this commercial role, *Lactobacilli* are important health enhancing flora of the gastrointestinal tract. Ideal pH conditions of 5.0 to 7.0 will permit*Lactobacilli* bacteria to compete with pathogenic microbes for essential nutrients and attachment sites. There are three strains of *Lactobacillus acidophilus* species involved maintaining normal intestinal flora and for preventing growth of undesirable bacteria. While all three of these organisms are able to tolerate an acidity within a pH range of 4.0 to 8.0. The growth of *S.aureus* in foods presents a potential public health hazard, since many strains of *S.aureus* produce enterotoxins that causes food poisoning if ingested (Varnam and Suitherland, 1995). Some of the strains of *Lactobacillus* produced bacteriocin type substances which inhibited the Gram negative bacteria tested, viz. *Pseudomonas sp.,E.coli*, and *S. typhimurium*. The target of bacteriocin is the cytoplasmic membrane, so due to the protective barrier provided by the LPS (Lipopolysaccharide) of the outer membrane of Gram negative bacteria, Bacteriocins are generally only active against Gram positive cells (Stevens *et al*., 1991). *L.acidophilus* was able to inhibit the growth of *E. coli*, *S. aureus*, and *Klebsiella sp.*. Inhibition zones were shown to be produced by the low pH of the *Lactobacilli* supernatants, as they disappeared when the supernatants were neutralized. Effect of pH on activity of bacteriocin was carried out by Ogunbanwo *et al*. (2003). It was observed that bacteriocin produced by *L.brevis* was stable at pH2 to pH8, while for *L.plantarum*, it was found to be stable at pH2 to 6. The activity of bacteriocin elaboborated by the test isolates was also pH dependent. The highest antibacterial activity was exhibited in an acidic pH range of 2 to 6, while inactivation occurred at pH 8 to 12 (Ogunbanwo *et al*., 2003).

CONCLUSION

From the above study it can be concluded that all the five isolates of *L. acidophilus* were showing sufficient tolerance at different pH and bile salt concentration and were also showing remarkable antimicrobial activity against enteric pathogens. On the basis of these criteria, the isolate can be used as probiotic organisms. Out of all the five isolates (LA-1, LA-2, LA-3, LA-4 & LA-5). LA-3 isolate can be considered as best isolates. Because LA-3 isolate was most acid tolerant at high acidic condition *i.e.* pH 2 and most bile tolerant at 0.2% for 6 hour incubation and it was showing maximum antimicrobial activity at different pH level and different concentration of bile

salt against tested pathogens. These isolate can be recommended for the production of various dairy products. All the isolates were found to be sensitive for all tested antibiotics with slight variations. LA-2 was found to be most resistant isolate and LA-3 and LA-5 was found to be most susceptible isolates. From the present study it can be concluded that the *L.acidophilus* isolates can tolerate high acidic condition, differentbile salt concentration and can inhibit the growth of enteric pathogens. The conclusion of the study suggests that all isolates can act as probiotic, and are helpful in the improvement of our intestinal flora. Consumption of milk products containing such probiotic organisms can help in protecting from occurrence of common gastro intestinal infections. The verified antimicrobial activity of probiotic supports the development of this functional food as a key to the improvement of the health of the consumers and use as food preservatives contents in different packed food products. Though the present study reveals that the preisolated *Lactobacillus acidophilus* isolates, *i.e.* LA-3 is good organism. The further studies in future should be focused on the mechanisms of action of *L.acidophilus* isolate within the gastro intestinal tract and in the immune system which stimulate the *invivo* immunity effects, bile tolerance, acid tolerance, resistance against enzymes and antibiotic sensitivity pattern. Furthermore, molecular characterization, genetic engineering of probiotics and those newly discovered to make them more efficacious should be pursued.

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