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ASSESSMENT OF PROBIOTIC CHARACTERISTICS OF L.PLANTARUM

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ABSTRACT
The present study was conducted to assess the probiotic properties of Lactobacillus plantarum which was obtained from National Collection of Dairy Cultures (NCDC), Karnal, Haryana, for its incorporation in the preparation of a nutraceutical food. Basic identification of Lactobacillus plantarum was done based on phenotypic characters *viz*., cell morphology, catalase, citrate utilization, tolerance to salinity and carbohydrate fermentation profile. L.*plantarum* was then screened for probiotic properties like bile tolerance, tolerance to acidity and inhibitory/antimicrobial activity. It was observed that L.plantarum showed tolerance to a maximum bile concentration of 0.6 per cent with cell viability of 8.470 ± 0.015 log10cfu/ml and tolerance to acidity at pH 3 with cell viability of 8.626 ± 0.106 log10cfu/gm. Also, L.*plantarum* showed maximum antimicrobial activity against Salmonella (zi 3.083 ± 0.040 mm), Staphylococcus aureus (zi 2.200 ± 0.100 mm) and Escherichia coli (zi 1.500 ± 0.025 mm). The results of the present study indicate that Lactobacillus plantarum exhibit probiotic properties and can be concluded to be used as a potential source of probiotic organism in the preparation of nutraceutical foods containing probiotics.

INTRODUCTION

At present, several well-characterized strains of Lactic Acid Bacteria and Bifidobacteria are available as potential probiotic organisms which are useful for improvising human health. The largest group of Lactic acid bacteria belongs to genus Lactobacillus that comprises more than 50 different species (Stiles and Holzapfel, 1997). Lactobacillus species are found in the gut of humans and other animals, while their numbers may vary with the animal species, age of host or their location within the gut. However, species of Lactobacillus like L.*acidophilus*, L.*crispatus*, L.*plantarum*, L.*gasseri* are involved in traditional and industrial food fermentations (De Vries *et al*., 2006).

The name probiotic comes from the Greek word "pro bios" which means "for life". The history of probiotics began with the history of man; cheese and fermented milk were well known to the Greeks and Romans, who recommended their consumption especially for children and convalescents. The concept of probiotics was introduced by Elie Metchnikoff in the early 20th century (Metchnikoff, 1907). According to the Food and Agriculture Organization (FAO) of the United States and World Health Organization (WHO), probiotics are 'live microorganisms which when administered in adequate amounts confer a health benefit to the host'. Alternatively, probiotics have been defined as live microbial feed supplements that beneficially affect the host animal by improving its intestinal microbial balance (Fuller, 1989).

The primary clinical interest in the application of probiotics in foods has been in the prevention of and treatment of GI infections and diseases (Parvez *et al*., 2006). Williams (2010) reported that probiotics exert their beneficial effects through various mechanisms, including lowering intestinal pH, decreasing colonization and invasion by pathogenic organisms, and modifying the host immune response. The FAO / WTO draft guidelines (2002) and ICMR-DBT guidelines for evaluation of probiotics in food (2011) recommended tolerance to bile salts, resistance to gastric acidity and antimicrobial activity against potentially pathogenic microorganisms as some of the attributes of potential probiotic organisms. An essential condition to select a particular probiotic strain is their ability to survive the transit through small intestine and the tolerance towards bile salts (Charteris *et al*., 1998). Gilliland *et al*. (1984) opined that though the bile concentration of the human gastrointestinal tract varies, the mean intestinal bile concentration is believed to be 0.3 per cent w/v. Goldin *et al*. (1992) reported that a concentration of 0.3 per cent w/v of bile was used in most screening studies for bile resistant strains. Garriga *et al*. (1998) opined that selection of bile tolerant strains at bile concentration between 0.1 and 0.4 per cent w/v in growth is typical.

Acid tolerance is another important quality a probiotic strain should possess because the gastric pH frequently falls below 2.0 (McLauchlan *et al*., 1998). Prasad *et al*. (1998) subjected Lactobacillus cultures between pH 1 to 3 and enumerated their growth after incubation at 37ºC. Hoque *et al*. (2010) reported in their study that the Lactobacillus species isolated from two regional yoghurts in Bangladesh was able to survive gastric environment at low pH (2.2). Morsy *et al*. (2015) evaluated antimicrobial activity of *L.plantarum* against seven

pathogens namely B.cereus, S.aureus, L.monocytogenes, E.coli, K.pneumoneae, P.aeruginosa and S.typhimurium and observed a zone of inhibition measuring 8.0, 9.0, 12.5, 8.0, 7.0, 8.0 and 8.5 mm diameter respectively. Hence, keeping in view the importance of the probiotic organisms in contribution to health benefits, the present work was performed to assess the probiotic characterization of *L.plantarum* proving it to be a potential probiotic that can be used in the preparation of nutraceutical food.

MATERIALS AND METHODS

Freeze dried culture of *Lactobacillus plantarum* was purchased from National Dairy Research Institute, Karnal, Haryana. De Man Rogosa and Sharpe (MRS) broth (Himedia GM369) was used for the propagation of the freeze dried culture and enumeration was carried out using De Man Rogosa and Sharpe (MRS) Agar (Himedia M641). Gram's staining (Himedia kit K001) of *L. plantarum* species was done and viewed using Nikon Model YS100 Binocular Microscope. Catalase test was performed as per slide method using 3% hydrogen peroxide solution. Citrate utilization test was performed using Simmons Citrate agar. Sugars used for carbohydrate fermentation test were Melibiose, Dextrose, Raffinose, Sorbitol, Mannitol, Sucrose, Fructose and Mannose (Himedia K008). Tolerance towards salt conditions was found at 2, 4, 6, 8 and 10% NaCl concentrations. Bile salt (make: Loba Chemie) was used to study the bile tolerance property. Tolerance of *L.plantarum* to acidity was tested using 5N HCl (Merck Millipore). Inhibitory activity of L.*plantarum* species was tested on Muller Hinton (MH) Agar with the pathogenic/ contaminating organisms Salmonella (isolated strain), Staphylococcus aureus (MTCC 96) and Escherichia coli strain (MTCC 452) maintained at Department of Veterinary Microbiology, Madras Veterinary College.

Propagation of freeze dried culture Lactobacillus *plantarum*

The freeze dried culture of *L.plantarum* was inoculated in MRS broth and incubated overnight at 37ºC. The activated culture in MRS broth was propagated twice prior to confirmation and characterization. The propagated stock culture was then enumerated using MRS Agar. Stock cultures were maintained by sub-culturing once in 15 days.

Morphological and biochemical characteristics of *Lactobacillus plantarum*

Confirmation by Gram's staining

Gram stained smear of L.*plantarum* was phenotypically identified as gram positive rods under microscope.

Catalase test

The catalase test was performed as per slide method. Using an inoculating needle, culture from a well-isolated colony was placed onto a clean glass slide. A drop of 3 percent hydrogen peroxide solution was added to this culture and closely observed for the evolution of bubbles (Nikita *et al*., 2012).

Citrate utilization test

L.plantarum strain was seeded on the Simmons Citrate agar dispensed into inclined tubes, and then incubated at 37ºC for 48 hrs. The change of the medium colour to blue indicates a positive reaction, and if the medium remains greenish, the test is denoted as negative (Rhaiem *et al*., 2016).

Carbohydrate fermentation test

Inoculate the test organisms in peptone water sugar medium or broth based sugar medium containing carbohydrate discs and then incubate at 37ºC for up to 7 days. Examine daily for acid and gas production. In case of fermentation, the colour of sugar changes from red to yellow, reflecting the test as positive. The carbohydrate discs used were Sucrose, Fructose, Dextrose, Raffinose, Melibiose, Mannose, Sorbitol and Mannitol (Yadav *et al*., 1993).

Tolerance to salinity

For testing tolerance towards salinity, test tubes containing MRS broth were added with different concentrations (2, 4, 6, 8 and 10%) of NaCl. After sterilization, each test tube was inoculated with 1 percent (v/v) fresh active culture of L.*plantarum* and incubated at 37º C for 24 hours. After incubation their growth was determined by observing their turbidity. Maximum growth were indicated as double positive sign (+ +), normal growth as single positive sign (+) and no growth as negative sign (Hoque *et al*., 2010).

Screening of Lactobacillus plantarum for probiotic properties

Tolerance of *Lactobacillus plantarum* to bile salts

Tolerance of *L.plantarum* to bile salts was carried out as per the method adopted by Sharma *et al*. (2013). *L.plantarum* was cultured in MRS broth containing different percentages of bile salt (0.2, 0.4 and 0.6 per cent w/v). Freshly prepared cultures were inoculated (1%) into medium and incubated at 37ºC for 24 hours under anaerobic condition. The tolerance of *L.plantarum* was evaluated by enumerating the colonies on petri dishes after incubation at 37ºC for 24 hours.

Tolerance of Lactobacillus plantarum to acidity

The tolerance of *L.plantarum* to acidity was evaluated by the enumeration of cells after incubation of the bacteria in MRS broth adjusted to pH 3 with HCl (5N) at 0, 90 and 180 mins (Zinedine and Faid, 2007).

Inhibitory activity test against pathogenic/contaminating bacteria

Inhibitory activity screening of *L.plantarum* was detected by agar well diffusion method on Muller Hinton (MH) agar against following bacterial cultures: Staphylococcus aureus (MTCC 96), Salmonella (isolated) species and Escherichia coli (MTCC 452). The bacterial cultures were inoculated on MH agar plates using sterilized cotton swabs. In each of these plates, wells were made using a sterilized gel borer. The 100 μl of *L.plantarum* inoculum were loaded into each well. Plates were incubated at 37ºC for 24 hours. After incubation, all plates were examined for the presence of zone of inhibition around the wells (Sharma *et al*., 2013).

Statistical analysis

The data obtained were analysed statistically as per the procedure of Snedecor and Cochran (1980).

RESULTS AND DISCUSSION

Propagation of freeze dried culture Lactobacillus plantarum

The freeze dried culture of *L.plantarum* was revived using

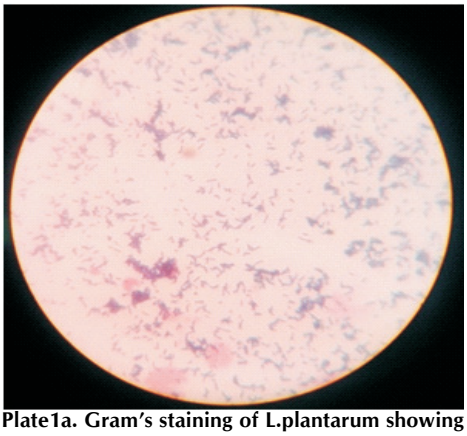

Plate1a. Gram's staining of L.plantarum showing gram positive cell morphology rods

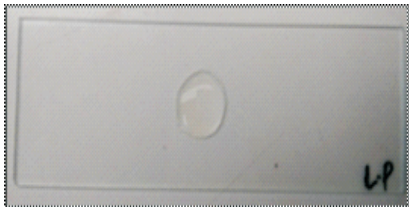

Plate 1b. Catalase test showing negative for *L.plantarum*

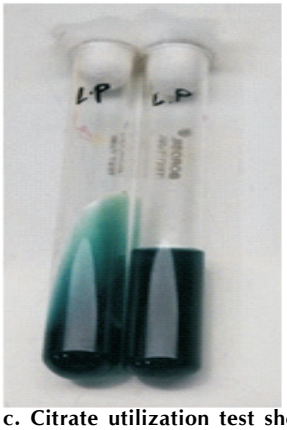

Plate 1c. Citrate utilization test showing negative for *L.plantarum*

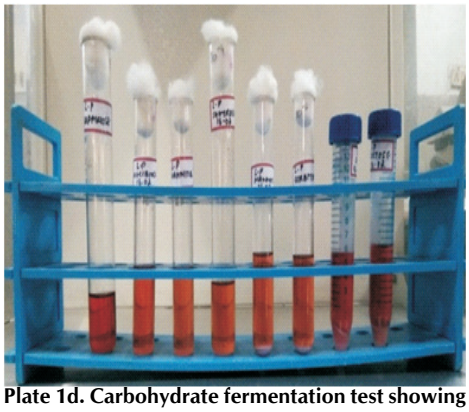

Plate 1d. Carbohydrate fermentation test showing positive for all sugars

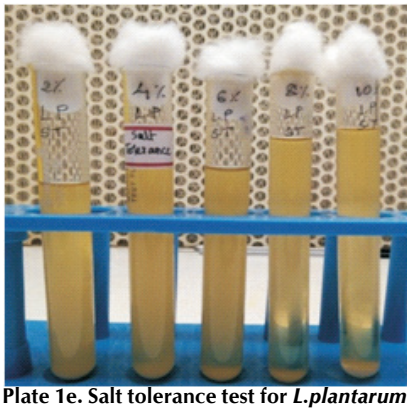

Plate 1e. Salt tolerance test for *L.plantarum* showing positive

Table 1: Morphological and biochemical characteristics of Lactobacillus plantarum

Morphology	Cream- white circular
Gram staining	Gram positive rods
Catalase	–
Citrate Utilization	–
Acid from	
Sucrose	+
Fructose	+
Dextrose	+
Raffinose	+
Mannose	+
Melibiose	+
Sorbitol	+
Mannitol	+
Salt tolerance (%)	
2	+ +
4	+
6	+
8	+
10	+

- Negative reaction; + Positive reaction

MRS broth and enumerated by pour plate method using MRS agar where the initial cell concentration was found to be 10.964 ± 0.133 log10cfu/ml.

Morphological and biochemical characteristics of *Lactobacillus plantarum*

Table 1 shows the morphological and biochemical characteristics of *L.plantarum*. The morphology of *L.plantarum* is shown as cream- white circular colonies. Gram positive *L.plantarum* rods are shown in Plate 1a. The cell morphology in the present study depict various cell morphology patterns and is concurrent with the findings of Chowdhury *et al*. (2012) who described *L.plantarum* as rod-shaped gram positive bacterium with the colony morphology showing round shape, off white to cream colour shiny colonies. Rhaiem *et al*. (2016) also affirmed the gram positive, rod shaped morphology of *L.plantarum* as observed in the present study. Similar phenotypic characterization was performed by Narayanan *et al*. (2017) for the preparation of ragi millet based probiotic food containing Bifidobacteria as the potential probiotic source, after isolating Bifidobacterium longum from infant faeces.

The ability of *L.plantarum* to show effervescence with hydrogen peroxide due to the presence of catalase enzyme and their ability to use citrate as the prime source of carbon is tested using catalase and citrate utilization tests, for which the organism showed negative result (Plates 1b and 1c), indicating gram positive organism. The ability of *L.plantarum* to metabolize different types of carbohydrates has been used for identification purposes and Table 1 and Plate 1d illustrates that *L.plantarum* was able to ferment the sugars sucrose, fructose, dextrose, raffinose, mannose, melibiose, sorbitol and mannitol. Also the ability of *L.plantarum* to grow in high saline

Table 2: Tolerance of Lactobacillus plantarum# to bile salts (Mean ± SE)@

Name of the culture	Control	Percentage of bile salt			F value
		0.20%	0.40%	0.60%	
Lactobacillus plantarum	10.964d ± 0.133	10.273c ± 0.065	9.167b ± 0.038	8.470a ± 0.015	208.29**

@Average of six trials; #log10cfu/ml

** Statistically highly significant (P ≤ 0.01); Means bearing various superscripts in the same row differs highly significantly (P ≤ 0.01); Small case shows significant difference between treatments

Table 3: Tolerance of Lactobacillus plantarum# to acidity at pH 3 (Mean ± SE)@

Name of the culture	Incubation period in minutes			F value
	0	90	180	
Lactobacillus plantarum	10.964c ± 0.133	9.967b ± 0.042	8.626a ± 0.106	133.49**

@Average of six trials; #log10cfu/ml

** Statistically highly significant (P 0.01);Means bearing various superscripts in the same row differs highly significantly (P ≤ 0.01); Small case shows significant difference between treatments

Table 4: Inhibitory activity of Lactobacillus plantarum (inhibition zone radius in mm) against pathogenic bacteria@

Name of the culture	Salmonella species		Staphylococcus aureus		Escherichia coli	
	CFS	CFS pH7	CFS	CFS pH7	CFS	CFS pH7
Lactobacillus plantarum (zi)	3.083 ± 0.040	2.583 ± 0.070	2.200 ± 0.100	1.583 ± 0.047	1.500 ± 0.025	0.500 ± 0.025

@Average of six trials; CFS- Cell free supernatant; CFS pH7- Cell free supernatant adjusted to pH7; zi- zone of inhibition

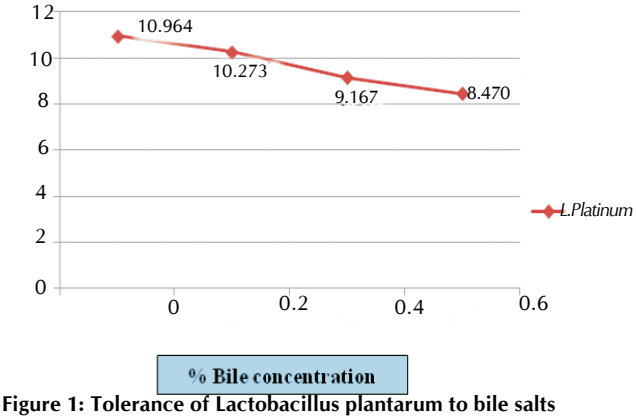

Figure 1: Tolerance of Lactobacillus plantarum to bile salts

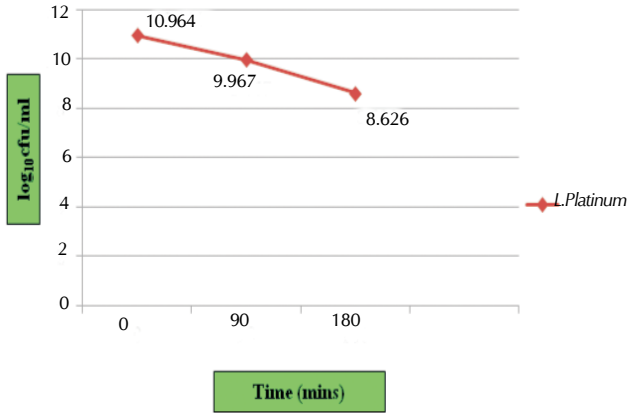

Figure 2: Tolerance of Lactobacillus plantarum to acidity at pH 3

environments has been used for identification purposes and Table 1 and Plate 1e illustrates that *L.plantarum* was able to grow in salt conditions of up to even 10 per cent, with highest tolerance shown in 2 per cent salt concentration compared to 4, 6, 8 and 10 per cent salt concentrations. The biochemical characterization in the present study is concurrent with the results obtained by Chowdhury *et al*. (2012) who reported negative for catalase and citrate utilization test and positive for the sugars glucose, xylose, sucrose, fructose, galactose, lactose, maltose, trehalose, ribose, rhamnose, mannitol and dextrose for *L.plantarum*.

Screening of Lactobacillus plantarum for probiotic properties

Tolerance of Lactobacillus plantarum to bile salts

From Table 2, Figure 1 and Plate 2, it was seen that the *L. plantarum* had tolerance towards varying levels of bile. *L. plantarum* showed higher tolerance (P ≤ 0.01) to 0.2 per cent of bile compared to 0.4 and 0.6 per cent of bile. Though there was highly significant difference in the tolerance of *L. plantarum* in increasing level of bile, *L. plantarum* showed considerable viable count at 0.4 and 0.6 per cent bile concentrations. The mean ± SE viable count (log10cfu/ml) at 0.4 and 0.6 per cent of bile were 9.167 ± 0.038 and 8.470 ± 0.015 respectively. The growth of *L. plantarum* at different concentrations of bile is affirmed by Sharma *et al*. (2013) who reported that all the isolates of Lactobacillus species collected from homemade curd samples were able to survive over a range of 0.05 - 0.3 percent w/v supplementation of bile-salt in MRS broth. This was further affirmed by Zinedine and Faid (2007) who reported that the Bifidobacterium belonging to LAB showed tolerance to 0.3 per cent bile. In the present study bile concentration of 0.2, 0.4 and 0.6 per cent w/v were used. However the mean intestinal bile concentration is believed to be 0.3 per cent w/v as reported by Gilliland *et al*. (1984). The use of 0.2, 0.4 and 0.6 per cent of bile was also in agreement with Goldin *et al*. (1992) who reported that the concentration of 0.3% (w/v) of bile has consequently being used in more studies screening for bile tolerance strains.

According to FSSR regulations (2016), the viable number of organisms in food with added probiotic ingredients shall be ≥ 10^8 cfu/gm. In the present study tolerance of *L. plantarum* to 0.4 and 0.6 per cent of bile was highly significant (P ≤ 0.01) from 0.2 per cent, maintaining minimum prescribed viable counts at all concentrations of bile as suggested by FSSR. Hence, the tolerance to bile was in tandem to the recommendation by FAO/WHO guidelines and ICMR –DBT guidelines for evaluation of probiotics (2011) to fulfill the attributes of a potential probiotic.Inhibitory activity of Lactobacillus

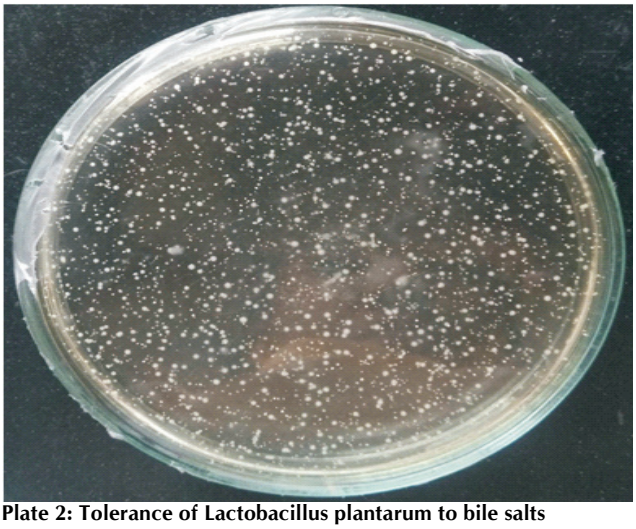

Plate 2: Tolerance of Lactobacillus plantarum to bile salts

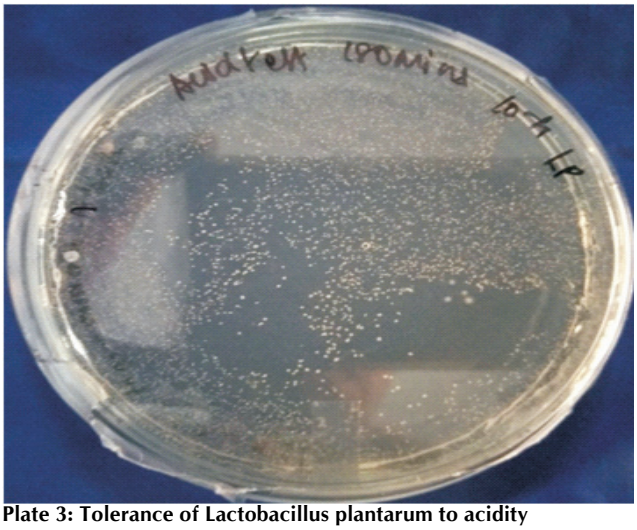

Plate 3: Tolerance of Lactobacillus plantarum to acidity

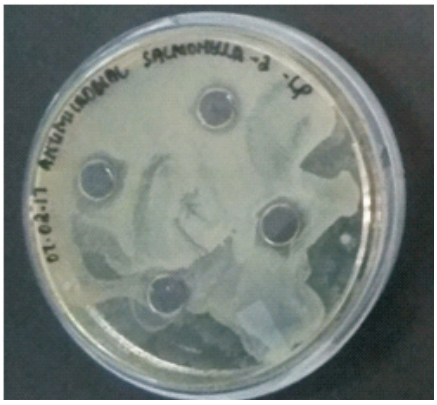

Plate 4a. *L.plantarum* showing inhibitory action against *Salmonella*

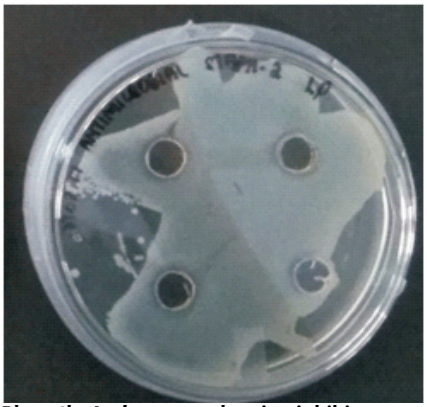

Plate 4b. *L.plantarum* showing inhibitory action against *Staphylococcus*

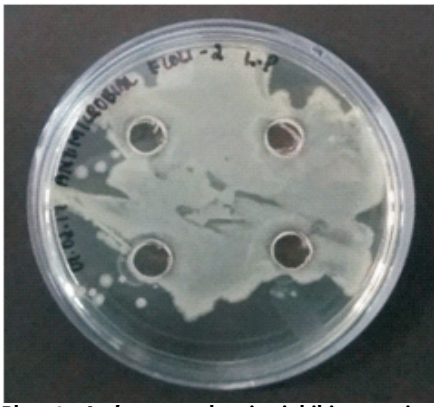

Plate 4c. *L.plantarum* showing inhibitory action against *Escherichia coli*

plantarum isolates against pathogenic bacteria

Tolerance of Lactobacillus plantarum to acidity at pH 3

Table 3, Figure 2 and Plate 3 showed the tolerance of *L. plantarum* to acidity at pH 3 as seen in most in vitro assays. This is affirmed by Prasad *et al*. (1998) who subjected Lactobacillus cultures to acidity between pH 1 to 3.

The mean ± SE viability at 90 mins was 9.967±0.042 log10cfu/ml. This was in agreement to Salminen *et al*. (1998) that for probiotics strains to survive and colonize in the gastrointestinal tract, they must express tolerance to acidity. In the present study marginal decrease was noticed in the viable count of *L. plantarum* at pH 3. However it maintained a viable count of 10^8cfu/ml after 180 minutes of incubation at pH 3. Hence this was in agreement to the recommendation of FAO/ WHO guidelines of 2002 and ICMR-DBT guidelines for evaluating probiotics (2011), where tolerance to gastric acidity is one of the attributes of a potential probiotic. The findings were also in consonance to the observations of McLauchlan *et al*. (1998) that acid tolerance is an important quality for a probiotic.

Inhibitory activity against pathogenic/contaminating bacteria

From Table 4 and Plates 4a, 4b and 4c, it is evident that the non-neutralized and neutralized cell free supernatants of *L. plantarum* showed inhibition against three among four of the pathogenic/ contaminating species tested.

In the present study *L. plantarum* showed inhibitory activity against selected contaminating bacteria. On comparing the inhibitory assay, it is noted that non-neutralized *L. plantarum* had a maximum inhibitory zone of 3.08, 2.20 and 1.50 mm against Salmonella, S. aureus and E.coli respectively due to acid and antibacterial compounds which was similar to the work of Morsy *et al*. (2015) who evaluated antimicrobial activity of *L.plantarum* against seven pathogens namely *B.cereus*, *S.aureus*, *L.monocytogenes*, *E.coli*, *K.pneumoneae*, *P.aeruginosa* and *S.typhimurium*. The results in the present study were similar to the work of Lade *et al*. (2007) where the Lactic acid producing bacteria (LAB) isolated from spoiled vegetables and curd were screened for the production of bacteriocin and were identified to be *L.lactis*, *L.plantarum* and *L.acidophilus*, which showed inhibitory activity against Escherichia coli.

The findings in the present study also met the requisites of antimicrobial activity against potentially pathogenic microorganisms as per the FAO / WTO draft guidelines of 2002 and ICMR-DBT guidelines 2011 for evaluation of probiotics in food. Hence the zone of inhibition shown by *L.*

plantarum against pathogenic species indicates that it can be used as a potential probiotic source in the preparation of nutraceutical and probiotic foods.

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