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Evaluation of probiotic potential of *Lactobacillus* isolated from indigenous plant sources

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Abstract

Probiotics are non-pathogenic, live micro-organisms which confer beneficial effects to host when administered in adequate amount. *Lactococcus* and *Lactobacilli* are representative genera of probiotic lactic acid bacteria (LAB). The present study was conducted to isolate *Lactobacillus species* from indigenous plant sources, characterize them and evaluate their Probiotic potential. A total of 30, samples from different plant sources were processed for the isolation of *Lactobacilli*. Samples were divided in to three groups: Group I (fruits), Group II (vegetables) and Group III (miscellaneous). Out of 30 samples only 4 samples were positive for *Lactobacilli*. *Lactobacilli* were isolated on de Man Ragosa and Sharpe (MRS) agar and identified on the basis of macroscopic, microscopic observations and biochemical tests. Isolates from strawberry, tomato and coriander samples were identified as *Lactobacillus plantarum*. One of the isolates from tomato was identified up to genus level only. Probiotic potentials such as resistance to low pH, bile salt and gastric juice were evaluated by viable colony count. Antibiotic susceptibility testing (AST) and antimicrobial activity were evaluated by agar diffusion methods. Isolates were resistant to pH 4, bile salt and gastric juice. Isolates were resistant to Penicillin G, susceptible to Sulfamethoxazole trimethoprim, gave intermediate results against Oxytetracycline and variable results against Pipemidic acid. All the indicator pathogens were significantly inhibited by the isolates ($P< 0.05$). The results of study revealed that *L. plantarum* is most abundant *Lactobacillus* isolated from indigenous plant sources. Indigenous fruits are good source of *Lactobacillus* than indigenous vegetables. *L. plantarum* showed good probiotic potential.

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Introduction

Probiotics are "Live micro-organisms which when administered in adequate amounts confer a health benefits on the host" (FAO/WHO, 2001). Majority of the probiotics used are members of "*Bifidobacterium* and Lactic acid bacteria (LAB)" (Pundir *et al.,* 2013).

Some of the health benefits confer by LAB are resistance against infectious diseases, improvement of immune status, reduction of cholesterol level, prevention of colon cancer and protection against radiation-induced intestinal injury (Demirer *et al.,* 2007; Balamurugan *et al.,* 2014). *Lactobacillus*, is one of the representative genus of LAB. Members of genus *Lactobacillus* are gram positive, non-spore forming, rods or coccobacilli. They are usually catalase negative, however sometimes some strains show pseudo-catalase activity. They are microaerophilic, fermentative and chemo-organotrophic. They require rich media for growth. Members of genus *Lactobacillus* are ubiquitous in nature found in carbohydrate rich environment such as dairy products, sour doughs, fermented meat, fruits, vegetables and beverages. They are also found in Gastro-intestinal tract (GIT), respiratory and genital tract of animals and humans, in plant materials and sewage (Felis and Dellaglio, 2007). Use of plants for medical purpose has a far back history. Plants can be used to treat different diseases by different mode of consumption. Fruit of *Solanum torvum* (Pea eggplant) is used to prevent typhoid, leucorrhoea and tonsillitis. Antimicrobial activities and antiviral activities have been showed by methanolic extract of fruit of *S. torvum* (Sofowora *et al.,* 2013*;* Yousaf *et al.,* 2013).

In Pakistan, gastrointestinal problems like cholera, diarrhea and dysentery are treated by various medicinal plants. Diarrhea is treated by decoction of bark of *Albizia lebbeck* (Sirin). Diarrhea and dysentery are effectively treated by fruit of *Phyllantus emblica* (Amla). Diarrhea is also treated by decoction of bark *Acacia nilotica* (Kikar) (Tariq *et al.,* 2015).

Extract of *A. lebbeck* had shown moderate antimicrobial activity against *Aeromonas hydrophila, Vibrio cholera* and *Bacillus subtilis*.

It has been reported that phenolics and flavonoid possess antimicrobial activities. These compounds are present in crude methanolic extracts of *A. lebbeck*. In vitro testing of methanolic extract of *P. emblica* fruit showed inhibitory effect against *Escherichia coli* and *Shigella dysenteriae*. While it's phytochemical analysis showed the presence of saponins, phenols, glycosides, flavonoid, proteins and carbohydrates. The bark and leaf extract of *A. nilotica* showed in vitro antimicrobial activity against *E. coli* and *Staphylococcus aureus*. Phytochemical analysis showed that plant contains various secondary metabolites (Tariq *et al.,* 2015).

Although data related to phytochemical analysis of medicinally important plant extracts are available. However, very little attempts have been made, especially in Pakistan to isolate LAB from plant sources and use them as probiotics. The study was conducted to isolate *Lactobacillus* from indigenous plant sources and to evaluate their probiotic properties.

Materials and methods

Sample collection

Different plant samples: Fruits, vegetables, Aloe Vera gel, and leaves of various fruit trees were collected for isolation of *Lactobacillus species*. Fruits (Banana, Cucumber, Guava, Lemon, Strawberry and Tomato) and Vegetables (Cabbage, Coriander, Onion, Potato and Radish) samples were purchased from various markets of Faisalabad. Leaves of Guava, Jujube, Lemon, Mango and Orange trees were procured from fields of a village in Faisalabad. *Aloe vera* samples were collected from house plant. Samples were collected in sterile plastic bags and transported to Probiotics Laboratory, Institute of Microbiology, University of Agriculture Faisalabad for further processing. Samples were washed with sterile distilled water. Washed samples were chopped into small pieces by sterile cutter (Trias *et al.*, 2008). However, lemon was squeezed for juice. The samples were kept at 4°C in sterile sample holders till further use.

Isolation and identification of Lactobacillus species

For isolation of *Lactobacillus species*, 10g of each chopped sample was weighed, triturated in sterilized

Motor and pastel and then dissolved into 90ml of normal saline (NS). Dissolving of samples was followed by homogenously shaking.

For broth enrichment 1ml of dissolved sample was inoculated into 9ml of de Man, Rogosa and Sharpe (MRS) broth. However, 1ml of each lemon juice and Aloe Vera gel was directly inoculated into 9ml of MRS broth. Broth was incubated at 37°C for 24-48 hours. Ten folds serial dilutions were made from incubated broth followed by "Spread Plate method". Plates were incubated an-aerobically at 37°C for 48 hours. Growth on MRS agar plates was further purified by streak plate method, until pure growth was obtained. Pure colonies were streaked on MRS agar slant and incubated at 37°C for 24-48 hours. After incubation MRS agar slant was stored in refrigerator at 4°C for further analysis (Pundir *et al.* 2013). Isolates were identified on the basis of colony morphology, Grams staining, spore staining, catalase activity and biochemical tests: Indole, Methyl Red, Voges Proskauer and Citrate Utilization (IMViC) and Carbohydrate fermentation tests (Anacaros *et al.,* 2015).

Evaluation of Probiotic potential of Lactobacillus species

Isolated Lactobacilli were evaluated for

Resistance to low pH

MRS broth tubes of pH 2, 4 and 7 were inoculated by isolated *Lactobacilli*, and serially diluted. Tubes were incubated at 37°C for 24-48 hours. From each tube 1ml inoculum was transferred to MRS agar plates. Plates were incubated at 37°C for 48 hours. The survival of *Lactobacillus* was checked by plate count method and "Colony forming unit per milliliter (CFU/ml)" was calculated (Tambekar and Bhutada, 2010).

The CFU/ml was calculated as follow:

$$\text{CFU/ml} = \frac{Average\ no\ of\ colonies \times dilution\ factor}{volume\ plated}$$

Resistance to bile salt

MRS broth tubes, supplemented with "0.3% w/v of Oxgall" were inoculated by isolated *Lactobacilli* and serially diluted. Tubes were incubated at 37°C for 24-48 hours. From each tube 1ml inoculum was transferred to MRS agar plates. Plates were incubated at 37°C for 48 hours. The survival of *Lactobacillus* was checked by plate count method and CFU/ml was calculated (Tambekar and Bhutada, 2010).

Resistance to gastric Juice

The gastric juice was prepared freshly. The isolates were inoculated in MRS broth and incubated at 37°C for 24 hours. After 24 hours of incubation broth was centrifuged at 5000rpm for 10 minutes and pellet was re-suspended in sterile physiological saline and inoculated into stimulated gastric juice, followed by serial dilution. Resistance was assessed by plate count method at different time intervals (0 hours, 1.5 hours and 3 hours) of incubation (Shafakatullah and Chandra, 2015).

Lactose utilization

Sterilized 10 ml of fermentation media was taken in sterilized test tube. Bacterial culture was inoculated to tubes and incubated at 35°C for 24-48 hours. After incubation, results were observed for change of color from red to yellow (Ahmed and Kanwal, 2004).

Antibiotic Sensitivity testing

Antibiotic sensitivity testing (AST) against commonly used antibiotics was determined by disc diffusion technique (Tambekar and Bhutada, 2010).

Antimicrobial Activity

Agar well diffusion method was used to determine antimicrobial activity of *Lactobacillus* (Manzoor *et al.,* 2006).

Statistical analysis

Data was analysed by "One Way Anova and Tukey's test" by using Minitab 17.

Results and discussion

Isolation and identification of Lactobacillus species

A total of 4 bacteria were isolated from tomato, coriander and strawberry only (Table 1). They were gram positive rods, catalase negative, non-spore formers and oxidase negative (Fig. 1, Fig. 2. and Fig. 3.). Isolates were further identified by conventional biochemical methods according to "Bergey's Manual of Systematic Bacteriology".

Biochemical characterization identified three of the isolates as "*Lactobacillus plantarum*" and one of the isolates was identified up to genus level only (Table 2). Isolation of *L. plantarum* from, vegetables (Amin *et al.,* 2009; Bamidele *et al.,* 2011)various fruits of Pakistan (Naeem *et al.,* 2012) and African star apple (Lawal *et al.,* 2016) has also been reported.

Table 1. Plant Samples positive or negative for isolation of *Lactobacillus*.

Group I: Fruits		Group II: Vegetables		Group III: Miscellaneous	
Sample	Growth	Sample	Growth	Sample	Growth
Banana	Negative	Cabbage	Cocci	Aloe Vera gel	Negative
Cucumber	Negative	Coriander	Positive	Leaves of Guava tree	Negative
Guava	Negative	Onion	Negative	Leaves of Jujube Tree	Negative
Lemon	Negative	Potato	Negative	Leaves of Lemon tree	Negative
Tomato	Positive	Radish	Negative	Leaves of Mango tree	Negative
Strawberry	Positive			Leaves of Orange tree	Negative

Table 2. Microscopic and biochemical characterization of isolates.

Sr no.	Sample	Gram staining	IMViC test			
			Indole	Methyl Red	Voges Proskauer	Citrate Utilization
1	Coriander	+ rods	-	+	-	-
2	Tomato	+ rods	-	+	-	-
3	Tomato	+ rods	-	+	-	-
4	Strawberry	+ rods	-	+	-	-
	Carbohydrate fermentation profile					
	Glucose	Lactose	Sorbitol	Maltose	Mannitol	Isolates Identified
1	+	+	+	+	+	*L. plantarum* 1
2	+	+	+	+	+	*L. plantarum* 2
3	+	-	+	+	+	*Lactobacillus species*
4	+	+	+	+	+	*L. plantarum 3*

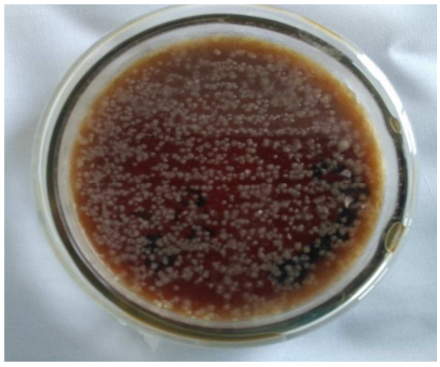

Fig. 1. Macroscopic observation of *Lactobacillus*.

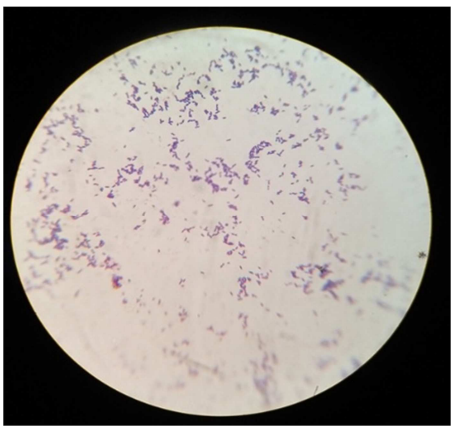

Fig. 2. Microscopic Observation of *Lactobacillus*. *Lactobacillus* appears purple (Gram Positive) rods.

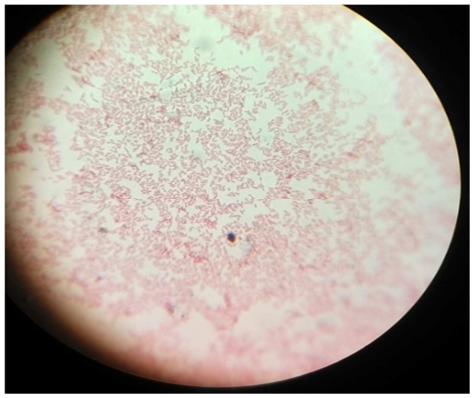

Fig. 3. Spore staining. *Lactobacillus* appeared red (Non-spore formers) after spore staining.

Evaluation of Probiotic Potential

In the present study, the isolates did not show survival at extreme acidic pH. No growth was observed at pH 2. However, isolates showed good growth at pH 4 and 7. The viable count at pH 4 and pH 7 was found to be significantly different ($P< 0.05$) (Table 3).

Garcia *et al.* (2016) reported that the counts of different strains of Lactobacillus, when exposed to pH 2.0 decreased sharply after 1 or 2 hours of incubation. Indeed, the count was not decreased after exposure to pH 5.0. All the isolates were resistant at 0.3% w/v of bile salt.

The viable count was not differed significantly (P> 0.05) at 0.0% w/v and 0.3% w/v of bile salt (Table 4). Shafakatullah and Chandra (2015) reported that LAB showed resistance to 0.3% and 0.5% bile salt. However, they did not survive at 1% concentration of bile salt. The viable count of isolates was decreased after 1.5 hours and 3 hours of incubation with gastric juice. The decrease in viable count was found to be significant (P< 0.05) (Table 5). Lactose utilization ability was shown by *L. plantarum* only (Table 2). To investigate the beneficial effects of indigenous plants and isolates on lactose intolerant people further in-vivo studies are required. Isolates was tested against "Penicillin G, Pipemidic acid, Sulfamethoxazole trimethoprim, Oxytetracycline" for antibiotic susceptibility testing. Isolates were susceptible to Sulfamethoxazole trimethoprim (25 µg). However, were resistant against Penicillin G (10µg). Isolates showed intermediate results against Oxytetracycline (30 µg) and variable results against Pipemidic acid (25µg) (Fig. 4. and Fig. 5.) The results of the study suggested that the antibiotic treatment with Penicillin G would probably not disturb Lactobacillus in GIT. Naeem *et al.* (2012) also observed high resistance in LAB isolated from fruit juices against "Oxacillin and Kanamycin" and hypothesized that use of Oxacillin and Kanamycin would not disturb LAB were tested for antimicrobial activity against "*E. coli, Klebsiella pneumonia, Pseudomonas aeruginosa, S. aureus*". *L. plantarum* exhibited varying degree of inhibitory activity against the indicator pathogenic bacterial strains (P< 0.05). *L. plantarum* showed highest zone of inhibitions against *E. coli* (Table 6). Amin *et al.* (2009) also reported that antimicrobial compound from *L. plantarum* and *L. casei* inhibited the growth of E. coli. In another study conducted by, Garzón *et al.* (2017) DNA sequencing confirmed that selected strains of "*L. plantarum* had structural gene "plw" to encode "plantacirin W".

Table 3. Resistance of isolates to low pH.

pH	*L. plantarum 1*	*L. plantarum 2*	*L. plantarum 3*	*Lactobacillus* species
	Log of (CFU/ml)			
4	5.43 ± 0.02^{A}	5.42 ± 0.04^{A}	5.45 ± 0.01^{A}	6.53 ± 0.05^{A}
7	7.53 ± 0.02^{B}	7.51 ± 0.03^{B}	7.54 ± 0.2^{B}	7.62 ± 0.03^{B}

Mean value ± Standard deviation of three trials. Results with-in column with different letters $^{A-B}$ are significantly different (P< 0.05).

Table 4. Resistance of isolates to bile salt.

Concentration of bile salt	*L. plantarum* 1	*L. plantarum* 2	*L. plantarum* 3	*Lactobacillus species*
		Log of (CFU/ml)		
0.0% w/v of bile salt	7.35 ± 0.04	7.21 ± 0.01	7.49 ± 0.03	7.33 ± 0.02
0.3% w/v of bile salt	7.34 ± 0.01	7.17 ± 0.03	7. 42 ± 0.03	7.31 ± 0.01

Mean value ± Standard deviation of three trials. Viable count at 0.0% w/v and 0.3% w/v of bile salt are not significantly different (P> 0.05).

Table 5. Resistance of isolates against gastric juice.

Incubation time	*L. plantarum* 1	*L. plantarum* 2	*L. plantarum* 3	*Lactobacillus species*
		Log of (CFU/ml)		
0 hours of incubation	7.31 ± 0.02^{A}	7.25 ± 0.04^{A}	7.45 ± 0.02^{A}	7.31 ± 0.01^{A}
1.5 hours of incubation	7.09 ± 0.03^{B}	7.03 ± 0.02^{B}	7.24 ± 0.01^{B}	7.01 ± 0.03^{B}
3 hours of incubation	6.70 ± 0.01^{C}	6. 72 ± 0.02^{C}	6.86 ± 0.03^{C}	6.69 ± 0.04^{C}

Mean value ± Standard deviation of three trials. Results within column with different letters $^{A-C}$ are significantly different (P< 0.05).

Table 6. Antimicrobial activity against pathogenic bacteria.

Indicator strains	*L. plantarum 1*	*L. plantarum* 2	*L. plantarum 3*	*Lactobacillus species*
E. coli	27.0 ± 2.0^{A}	18.0 ± 1.1^{A}	23.0 ± 2.08^{A}	10.0 ± 1.52
K. pneumonia	12.0 ± 1.0^{B}	8.0 ± 1.73^{B}	9.0 ± 0.58^{B}	R
P. aeruginosa	10.0 ± 1.52^{B}	11.0 ± 1.0^{B}	14.0 ± 1.15^{C}	R
S. aureus	R	R	R	R

Mean value (mm) ± Standard deviation of three trials. Results within column with different letters $^{A-C}$ differ significantly at (P< 0.05).

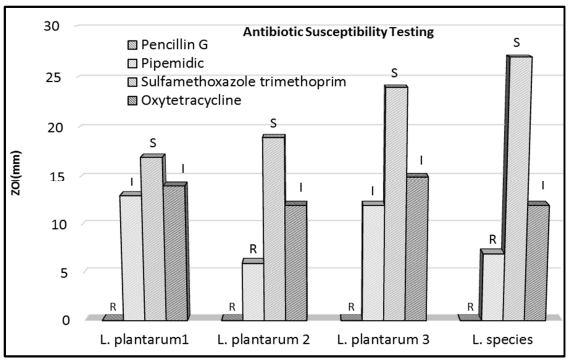

Fig. 4. Antibiotic susceptibility testing. ZOI stands for "Zone of inhibition".According to "National Committee for Clinical Laboratory Standards (NCCLS) for R (Resistant) ZOI≤ 12mm; I (Intermediate) ZOI= 13-14mm and S (Susceptible) ZOI≥ 15mm.

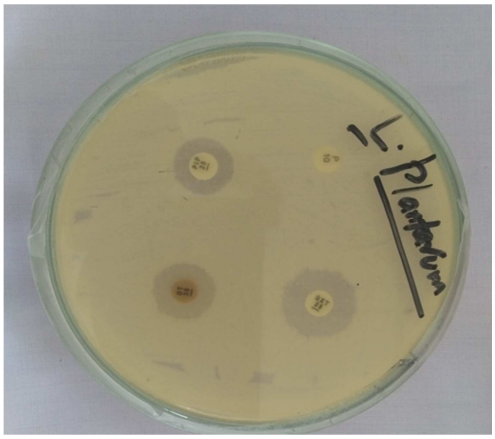

Fig. 5. Antibiotic Susceptibility testing.

The study concluded, *L. plantarum* is most abundant *Lactobacillus species* isolated from indigenous plant sources. Indigenous fruits are good source of *Lactobacillus* as compared to indigenous vegetables. *L. plantarum* showed good probiotic potential.

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