

*Research Paper***An Evaluation of *In-vitro* Potential of Novel *Lactobacillus Paraplantarum* KM0 (KX671558) Strain Isolated from Milk**

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Present study was carried out to evaluate probiotic potential of novel bacterial strain i.e., *L. paraplantarum* KM0 (accession number KX671558) isolated 1<sup>st</sup> time from pooled milk of Himachal Pradesh. *L. paraplantarum* KM0 exhibited antimicrobial activity against pathogenic food borne microorganisms (with 100% inhibition), highest viable counts at low acid value (i.e. at pH 2.0 and 3.0), 0.3% bile salt tolerance, 36.6% autoaggregation, co-aggregation ability (20.0%), 95% cell surface hydrophobicity against O-xylene and antibiotic susceptibility against most of the antibiotics used. All these desirable attributes make this strain a robust probiotic.

**Keywords:** Probiotics; *Lactobacillus paraplantarum* KM0; autoaggregation; antimicrobial activity; antibiotic sensitivity

**Introduction**

Milk is a rich source of potential microbial flora because milk is the only food that fulfils all the nutritional requirements of infants and adults. It also protects the baby against the pathogenic attack. Due to the presence of some components and prebiotics such as antimicrobial agents, immunocompetent cells and immunoglobulins, etc. (Martin *et al.*, 2003). The most commonly found beneficial microflora in milk is lactic acid bacteria which act as probiotics *in vitro*. Probiotics are known as health beneficial bacteria which contribute toward our well being by altering balance of intestinal flora, inhibiting food borne and other harmful pathogens, boosting immunity, improving digestion and increasing resistance to infection (Helland *et al.*, 2004). Probiotics also provides clinical benefits such as lowering of cholesterol, acting as anticarcinogens, increasing the bioavailability of nutrients, alleviation of intolerance to lactose and lower the risk of allergy, etc. (Parvez *et al.*, 2006).

The major topic of discussion regarding health is probiotics. Probiotics are defined by WHO/FAO as administration of living microorganisms by host in

adequate amount which confer health benefits upon ingestion (Fuller, 1989). Due to wide utility in biomedical field probiotics are used greatly in industries for the formation of novel nutraceutical and functional products.

Not much research has been done so far as regards isolation and evaluation of potential probiotic flora from different samples of the pooled milk of Himachal Pradesh (Martin *et al.*, 2003). Thus considering its importance in human health and also due to the growing research interest in lactic acid bacteria (LAB), the present study was undertaken to isolate and characterize probiotic microorganisms from pooled milk of Himachal Pradesh.

**Materials and Methods***Source of Culture*

KM0 culture was isolated from different samples of pooled milk (i.e. different human milks) of Himachal Pradesh. The culture was serially diluted and spread on sterilized petriplates containing solidified de Man, Rogosa and Sharpe (MRS media) at 37°C for 24h

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(Aneja, 2003). The culture of KM0 was maintained biweekly by transferring the culture into sterile litmus milk (of 1% concentration) for 24h at 37°C and followed by preservation at -4°C.

### Phenotypic and Biochemical Characterization

Under morphological and phenotypic characterization viz. color, texture, elevation and margin of colonies of KM0 are recorded. Biochemical characterization i.e. gram's staining, casein hydrolysis, carbohydrate fermentation, indole, MRVP, Citrate, Catalase, Mobility, H<sub>2</sub>S and gelatin hydrolysis test of isolated strain following standard microbiological techniques were performed (Aneja, 2003). Further tentative identification was carried out according to the criteria of Bergey's Manual of Determinative Bacteriology (7<sup>th</sup> edition) (Breed et al., 1957).

### Genomic Identification

Genomic DNA of KM0 was isolated according to the protocol given in DNA pre kit (Bangalore Genei, India Pvt. Ltd.). The PCR reaction mix included Taq buffer (10X)-5.0 µl; dNTPs-mix 2 mM-2.5 µl; primer (F)-10 µl; Taq polymerase- 2.0 µl; glycerol -0.5 µl; water-12.8 µl DNA-1 µl; MgCl<sub>2</sub>-1 µl. PCR was carried out with 35 cycles of 92°C for 1 min, 55°C 1 min, 72°C 1min. The universal primers used for amplification were BITS-1 (5'AGAGTTTGATCC TGG) and BITS-4 (5'TACCTTGTTACGACTT) which are expected to generate 1500 bp amplicon. The amplified PCR product was cleaned using the PCR clean-up kit (Real Genomics Hi Yield TM). Eluted PCR product of KM0 was sequenced by commercially available services of Xcelaris, Mumbai, India. The sequence homologies were analysed using the BLAST tool of NCBI (<http://www.ncbi.nlm.nih.gov/>).

On the basis of 16S rRNA sequence, the KM0 was identified as *Lactobacillus paraplantarum*. The sequences have been deposited at NCBI under the accession number KX671558. The phylogenetic tree of *L. paraplantarum* KM0 has been plotted using software MEGA 6 and presented in Fig. 1.

### Antimicrobial Activity

Serious food borne bacteria were used to study antimicrobial property against *Clostridium perfringens* MTCC 1739, *Staphylococcus aureus*

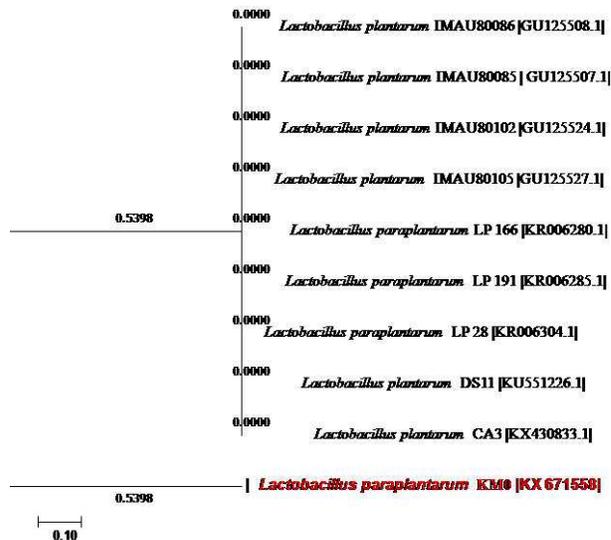


Fig 1: Phylogenetic tree of *Lactobacillus paraplantarum* KM0

IGMC, *Leuconostoc mesenteroids* MTCC 107, *Bacillus cereus* CRI, *Listeria monocytogens* MTCC 839 and *Enterococcus faecalis* MTCC 2729. These desired test indicators were obtained from IGMC, Shimla, H.P. India and IMTECH, Chandigarh, India. All the test indicators were revived after regular intervals of 15 days at 37°C for 24 h and preserved in 40% glycerol at -20°C. *Lactobacillus paraplantarum* KM0 culture showed antimicrobial activity against various test organisms using agar well diffusion assay as per the method of Kimura et al. (1998).

### Tolerance to Low pH

The acid tolerance of the culture was studied for different pH solutions. 100ml MRS broth solutions each were prepared by adjusting pH to 1.0, 2.0 and 3.0 by hydrochloric acid (0.1M HCl) solution. MRS broth with pH 6.5 served as a control. After thorough mixing, 10 ml of each pH solution was taken in sterilized test tubes. Culture was activated as mentioned above. Thereafter, bacterial cells were suspended in 10 ml of MRS broth @ 1% for 24 h. Cell suspensions containing about 10<sup>9</sup> cells/ml was added to each pH solutions of 1.0, 2.0 and 3.0 and control (pH 6.5) and mixed. At 0, 1, 2 and 3 h, 1ml from each pH solution was taken and dilutions of 0.1 % peptone water were prepared. The dilutions were spread on MRS agar and the plates were incubated for 24 h at 37°C. The viable cell counts were expressed in colony forming units (cfu/ml) (Gotcheva et al.,

2002).

#### **Ability to Tolerate Bile Salt Concentrations**

Bile salt resistance of the strain was examined following Lindgren and Dobrogosz (1990). The bile salt solutions were prepared using oxgall powder (HiMedia, India). The powder was rehydrated by preparing 10 g dry powder base in 90 ml distilled water (equivalent to rehydrated ox bile). From this solution, final concentrations of 1% and 2% were prepared in MRS broth and MRS broth without oxgall (pH 6.5) was used as control. The solutions were autoclaved and stored at room temperature till use. 10ml of each solution was transferred into sterile test tubes. Culture was activated properly. Thereafter, bacterial cells were finally suspended in 10 ml of their desired broth @ 1% for 24 h. Cell suspensions containing about 109 cells/ml were added to each solution, i.e., 1%, 2% and control and incubated at 37°C. At 0, 1, 2 and 3 h time intervals, 1 ml of culture was taken out immediately from each test tube and diluted in 0.1% peptone water to make 10 ml volume. Appropriate dilutions were spread on MRS agar plate and incubated for 24 h at 37°C aerobically. The viable cells counts were expressed in colony forming units (cfu/ml).

#### **Hydrophobic Property**

Ability of the organisms to adhere to hydrocarbons is a measure of their adherence to the epithelial cells in the gut is known as cell surface hydrophobicity and was determined according to the method described by Rosenberg *et al.* (1980) with slight modification using O-Xylene, chloroform and ethyl acetate. Thereafter, bacterial cells were suspended in 10 ml of MRS broth at the rate of 1% at 37°C for 18 h. Bacterial suspension was centrifuged and the pellet was washed twice with phosphate urea magnesium (PUM) buffer of pH 7.1. The pellet was resuspended in PUM buffer and the absorbance 610 nm was adjusted approx. 0.7-0.9 O.D. (Optical Density). *Lactobacilli* cell suspension (3.0 ml) containing O-xylene, chloroform and ethyl acetate (1.0 ml) were vortexed and incubated for 10 min at 37°C for equilibration of temperature. The mixture was remixed and incubated for 1 h at 37°C for separations of phase and the hydrocarbon layer was allowed to rise completely. After 1 h, aqueous phase was removed carefully with a pasteur pipette and the absorbance (A<sub>610nm</sub>) was measured using spectrophotometer

(JenwayGenova, Jenway Ltd. Gransmore Green, Felsted, Dunmow, UK). The decrease in the absorbance was taken as a measure of the cell surface hydrophobicity (%H) calculated as:

$$\text{Hydrophobicity \%} = [(A - A_0)/A] \times 100$$

where, A=initial absorbance and A<sub>0</sub>=final absorbance; with the three hydrocarbons at 610 nm.

#### **Cell Auto-aggregation**

The autoaggregation capacity of *L. paraplantarum* KM0 was determined as described by Kos *et al.* (2003). The culture was grown at 37°C for 18 h in MRS broth. The cell suspension was centrifuged at 10,000 rpm for 10 min and the pellet was washed twice with phosphate-buffered saline (PBS), pH 7.2 and re-suspended in the same buffer. Autoaggregation was calculated by measuring the absorbance at 0 h (A<sub>0</sub>) and after 5 h (A<sub>t</sub>) and calculated using the following formula:

$$\text{Autoaggregation (\%)} = [1 - (A_t/A_0)] \times 100.$$

#### **Cell Coaggregation**

Co-aggregation ability of each isolate was determined by following the method of Del Re *et al.* (2000) with some modifications. The isolate was inoculated into MRS broth and the indicators viz., *C. perfringens*, *B. cereus* and *L. monocytogens* were inoculated in nutrient broth and incubated for 24 h at 37°C. Cell suspension and pathogenic indicators were diluted to 1 OD ( $\lambda = 600\text{nm}$ ). Cell suspensions were centrifuged for 10 min at 10,000 rpm (at 4 °C) and the pellets were washed three times with 0.1 M PBS followed by re suspension in PBS. OD for isolate and indicator organisms was set to 0.5 ( $\lambda = 600\text{nm}$ ). To study the coaggregation ability, combinations of bacterial culture was made with all three test indicators individually (in the ratio 1:1). Separate test tubes of bacterial culture and indicator test strains were kept as control and incubated for 4 h at 35°C. Absorbance at wavelength of 600 nm were noted for mixture as well as for individual test strains. Co-aggregation % was calculated according to Handley *et al.* (1987).

$$\text{Coaggregation \%} = \{(A_{\text{PATH}} + A_{\text{LAB}})/2 - A_{\text{(MIX)}} / (A_{\text{PATH}} + A_{\text{LAB}})/2\} \times 100$$

### Antibiotic Sensitivity Assay

Susceptibility of selected *L. paraplantarum* KM0 culture to antibiotics was studied by disc diffusion assay as described by Clinical and Laboratory Standards Institute (CLSI, 2007). A total of 11 antibiotic discs (HiMedia Ltd, Mumbai, India) i.e. Amikacin (AK), Gentamycin (GEN), Ofloxacin (OF), Ampicillin (AMP), Erythromycin (E), Cefotaxime (CTX), Clindamycin (CD), Penicillin-G (P), Tetracycline (TE), Ciprofloxacin (CIP) and Cotrimaxazole were used. The zone of inhibition [in diameter (mm)] was measured by antibiotic zone scale and results were expressed in terms of resistance or susceptibility as given by Performance Standards for Antimicrobial Disk Susceptibility tests (CLSI, 2007).

### DNase Activity

DNase enzyme production of isolates was determined by following the method of Gupta and Malik (2007). Isolate was spot inoculated on the DNase agar medium (HiMedia) plates and then incubated at 37°C for 24-48 h. A clear pinkish zone around the colonies against dark blue background was considered as positive result for DNase enzyme production.

### Gelatinase Activity

Gelatinase activity of isolates was determined by following the method of Gupta and Malik (2007). Gelatinase enzyme production of isolates were determined by inoculating 20 µl of 12 h old culture on plates containing respective agar medium supplemented with 3% gelatin. The plates were incubated at 37°C for 24-48 h and then were flooded with saturated ammonium sulphate solution. Development of clear zones around the spots against the opaque background indicated a positive reaction.

### Haemolytic Activity

Haemolytic activity of the isolates were determined by spot inoculating overnight grown bacterial culture on Sheep Blood Agar plates supplemented with 5% human blood and incubated at 37°C for 24-48 h. Haemolytic activity of the isolates were examined for signs of  $\beta$ -haemolysis (clear zones around colonies),  $\alpha$ -haemolysis (green zones around colonies) or  $\gamma$ -haemolysis (no clear zones around colonies) on sheep blood agar medium plates (Harrigan, 1998).

## Result and Discussion

### Isolation and Identification of Probiotic Isolate

Isolate KM0 was identified up to genus level by morphological and biochemical characteristics. Morphologically KM0 colonies appeared cream coloured, punctiform with entire margin, raised elevation and smooth texture on MRS (Plate 1). Isolate KM0 was found to be catalase and gram positive bacilli. Biochemical tests viz., casein hydrolysis, indole production, MRVP test, and H<sub>2</sub>S production were found negative while fermentation of carbohydrates from glucose, motility test and citrate utilization were found positive for KM0 isolate. KM0 was identified following Bergey's Manual of Determinative Bacteriology as *Lactobacillus* sp. (Breed et al., 1957). The 16S rRNA gene technique was further used for species level identification. The sequence of KM0 was compared with the Genbank database. The 16S rRNA sequence of *L. paraplantarum* KM0 showed higher level of homology i.e. 96% with *L. paraplantarum* and registered under accession number KX671558 in NCBI and its phylogenetic tree is presented in (Fig. 1).



Plate 1: Morphology of *L. paraplantarum* KM0 on MRS agar

### Antagonistic Activity of KMO Against Food Borne Pathogens

The antimicrobial activity of *L. paraplantarum* KM0 against pathogenic microorganisms was determined by agar well diffusion assay. Antimicrobial activity

was determined by measuring the diameter of zone of inhibition (Plate 2), indicating that the *L. paraplantarum* KM0 culture was inhibitory against the test organisms. The zone of inhibition was found to range from 10 to 20 mm diameter. The isolate *L. paraplantarum* KM0 was most sensitive towards pathogen viz. *S. aureus* with diameter of zone of inhibition 20 mm which was significantly higher ( $P<0.05$ ) than others, followed by *L. monocytogenes* (18 mm), *B. cereus* (16 mm), *C. perfringens* (15 mm), *L. mesenteroides* (15 mm), *L. plantarum* (14 mm), *E. faecalis* (13 mm), *P. syringe* (13 mm) and *P. caratovorum* (13 mm). The antimicrobial activity of probiotic is thought to be significant criteria for full exclusion or inhibition of the activities of harmful intestinal microflora so that the host gets protection against these pathogenic microorganisms. Gautam and Sharma (2014) studied the antagonistic potential of *Lactobacillus brevis* UN isolated from Dulliachar-a salted pickle that exhibited broad spectrum activity against most of spoilage causing/food borne pathogens viz. *L. monocytogenes*, *C. perfringens*, *S. aureus*, *L. mesenteroides*, *L. plantarum* and *B. cereus*.

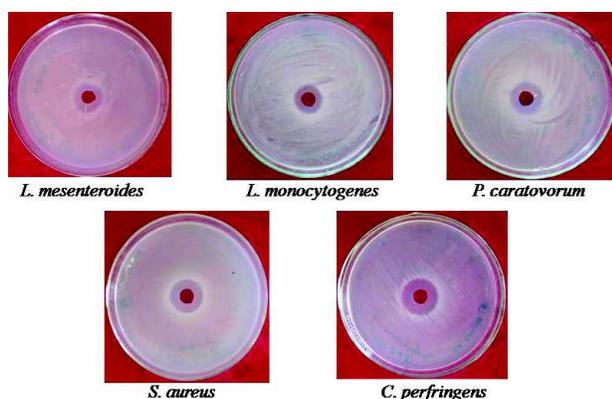


Plate 2: Antagonistic spectrum of *L. paraplantarum* KM0 by well diffusion method in terms of zone size

In human gastrointestinal tract, Lactobacilli are the natural inhabitants along with other microflora. These beneficial microorganisms inhibits multiplication of pathogens by producing organic acids (i.e. acetic and lactic acids), that lower the intestinal pH (Musikasang *et al.*, 2009). Probiotic microorganisms help in complete exclusion of pathogens from intestinal mucosa by co-aggregating with pathogenic organisms or by attaching to their enterocytes. The gram positive bacteria exhibit wide range of antimicrobial activity compared to gram negative because of presence of lipopolysaccharide (LPS) in their cell wall. In the present study, *L. paraplantarum* showed best antagonistic activity against both gram positive and gram negative food borne pathogens.

### Tolerance to Low pH

The effect of different pH on the viability of *L. paraplantarum* KM0 is presented in Table 1. *L. paraplantarum* KM0 culture were able to survive at pH 2.0 and pH 3.0 for 3h, with viability of 5.60 and 5.81 log counts respectively. The control is taken at 6.5 pH. KM0 was relatively more resistant at pH 3.0 for 3 h than pH 2.0 for 3 h and its survival is significantly higher ( $P<0.05$ ). Further, at pH 1.0, *L. paraplantarum* KM0 counts were found to rapidly decrease. In a similar type of work Gautam and Sharma (2015) observed that *L. spicheri* G2 had good tolerance power at pH 3.0 but after 1h at pH 1.0, a rapid decline was seen in viability with reduction of 5 log count.

### Tolerance to High Bile Concentrations

The bile salt concentrations in the gastrointestinal tract range from 0.5% to 2.5% in the first hour of digestion which decreases further in subsequent hours (Hati *et al.*, 2014). Hence, bile salt tolerance is con-sidered

Table 1: Acid tolerance of *L. paraplantarum* KM0

pH	Cell survival (log cfu/ml) Incubation time					% Cell Survival Incubation time			
	0	1	2	3	Mean	1	2	3	Mean
1.0	5.87	5.72	5.5	-	4.270	97.44	93.69	0.00	63.71
2.0	6.04	5.99	5.84	5.60	5.845	99.17	96.68	92.71	96.18667
3.0	6.16	6.08	5.97	5.81	6.005	98.70	96.91	94.31	96.64
Control (6.5)	6.30	6.20	6.14	6.08	6.180	98.41	97.46	96.50	97.45667
CD	Treatment (0.008)				Incubation Time (0.008)TxI (0.016)				

important criteria for colonization and metabolic activity of microorganisms in the small intestine of the host (Havenaar and Huis in't Veld 1992). In the present study from Table 2, it was depicted that the potential probiotic bacteria *L. paraplantarum* KM0 culture had survived in bile salts (0.3-2%) quite effectively. The culture had tolerated 0.3% bile with viability being 8.18 log counts after 8 h, however, viable counts differed significantly ( $P < 0.05$ ) in comparison to control. At higher concentration (2%), bile tolerance of the strain *L. paraplantarum* KM0 decreased marginally as compared to 0.3% bile after 8h. The *L. paraplantarum* KM0 culture exhibited slight increase in growth in control (media with 0% bile) with viability of 7 log cfu/ml after 3h. But after 8 h of incubations, viable counts differ significantly than 4 h of incubation at different bile concentrations. The resistance to bile salts varies a lot among the lactic acid bacteria species and even between strains themselves (Xanthopoulos, 1997). In similar studies, Fossi *et al.* (2015) showed survival of *L. plantarum* 1(D) and *L. pentosus* 1(G) at 3% (w/v) bile concentration with a survival rate of 75% for 2 h and dropping to 60% at 3 h. Martin *et al.* (2006) reported survival of *L. salivarius* CECT approximately 55% after exposition to conditions simulating these found in the gastrointestinal tract at pH 2.5.

### **Bacterial Adhesion to Solvents (Cell Hydrophobicity)**

The adherence to gut is an important criterion for probiotic bacteria. The probiotic organisms have the ability to adhere to the intestinal epithelium and colonize the human GIT (gastrointestinal tract) for exerting beneficial effects (Collado *et al.*, 2007; Licht and Wilcks, 2005). Adherence of bacterial cells is

usually related to their cell surface characteristics. Bacterial adhesion to solvents is a nonspecific interaction between microbial cells and host. Initially the interaction may be weak followed by more specific mechanisms including cell surface proteins and lipoteichoic acids. In vitro ability of probiotic microorganisms to adhere to epithelia is studied by cell surface hydrophobicity toward chloroform, O-xylene, and ethyl acetate. *L. paraplantarum* KM0 showed maximum adhesion of 95% towards O-xylene, 60% adhesion towards chloroform, and 40 % for ethyl acetate. A remarkably high adhesion for xylene (95%) proves that *L. paraplantarum* KM0 is strongly hydrophobic (Fig. 2). Microbial adhesion to chloroform, xylene and ethyl acetate is determined to assess the Lewis acid-base characteristics of the microbial cell surfaces. Out of three different solvents, xylene reflects cell surface hydrophobicity/hydrophilicity because it is apolar solvent whereas chloroform-a monopolar acidic solvent and ethyl acetate- a monopolar basic solvent are regarded as a measure of electron donor and electron acceptor.

The strain showed strong affinity to chloroform, which is an acidic solvent and electron acceptor, followed by ethyl acetate, which is a basic solvent and electron donor. In a similar study Gautam and Sharma (2015) showed that *L. spicheri* G2 displayed maximum affinity towards xylene (26 %) which is apolar solvent.

### **Autoaggregation Ability of *L. paraplantarum* KM0**

Autoaggregation ability is a nonspecific way to determine the capacity of the bacterial strain to interact with itself. Autoaggregation is one of the important factor to check the ability of the strain to adhere to

**Table 2: Bile tolerance of *L. paraplantarum* KM0**

	Bile salt (%)Cell survival (Log cfu/ml)				% Cell survival		
	Incubation time (h)				Incubation time (h)		
	0	4	8	Mean	4	8	Mean
Control (0.3)	8.26	8.22	8.18	8.22	99.51	99.03	99.27
0.7	8.18	8.21	8.24	8.21	99.87	99.14	99.505
1	8.18	8.17	8.07	8.14	99.87	98.65	99.26
1.5	8.17	8.14	8.03	8.11	99.63	98.28	98.955
2	8.13	8.08	7.99	8.06	99.38	98.27	98.825

CD Treatment (0.010) Incubation Time (0.007)TxI (0.016)

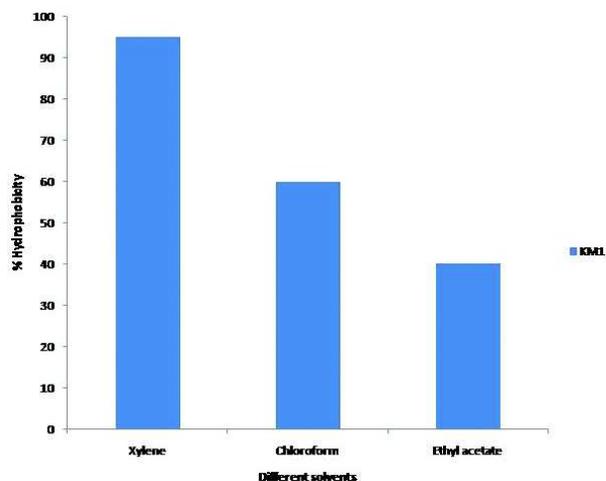


Fig. 2: Cell surface hydrophobicity of *L. paraplantarum* KM0 to selected hydrocarbons (xylene, chloroform and ethyl acetate)

the oral cavity and gastrointestinal tract. The rate of sedimentation in *L. paraplantarum* KM0 was measured upto 5 h. In the initial period, the percentage of autoaggregation was 1% that rise rapidly throughout the experiments and finally registered as high percentage as 36.6. Results showed that this strain has a strong autoaggregating property. This property could be related to cell surface component that is not lost after washing and suspension of cells in PBS (Fig 3). Similar results had been reported by Puniya *et al.* (2016) who measured sedimentation rate of four isolates viz. LH14, LH17, LK12 and LH12 that ranged between 48-73%. Vaz-Vilho and Todorov (2011) observed that the autoaggregation ability of *L. plantarum* ST16Pa was 37.05% after 60 min of incubation.

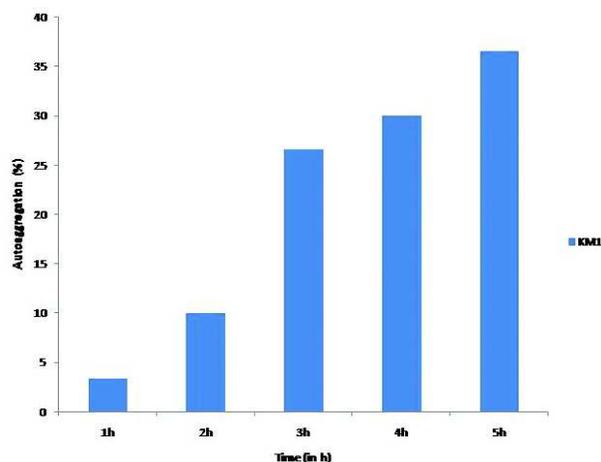


Fig. 3: Cell autoaggregation of *L. paraplantarum* KM0 at different time interval (1h, 2h, ..., 5h)

### Co-aggregation Ability

Probiotics effectively kill or inhibit wide range of pathogenic microorganisms by secreting antimicrobial compound and acting directly on the cells of pathogenic bacteria (Bao *et al.*, 2010). In this study, the coaggregation ability of *L. paraplantarum* KM0 was studied and its percent coaggregation ability with pathogens viz., *L. monocytogenes*, *C. perfringens* and *B. cereus* has been depicted in Fig. 4. *L. paraplantarum* KM0 exhibited highest co-aggregation potential with *L. monocytogenes* i.e., 20.0 % and lowest co-aggregation with *B. cereus* and *C. perfringens* i.e. 6.6 %. Similar observations have been reported by Li *et al.* (2015) who studied the co-aggregation ability of 18 lactic acid bacteria isolates taken from traditional fermented foods and found all the isolates to have co-aggregation ability with *Salmonella* sp. ranging from 5.15% to 29.54%. *L. fermentum* after 9 h showed the highest co-aggregation ability of 29.54% followed by *L. fermentum* AB4 (19.45%) while *E. faecalis* 5-5 showed the lowest co-aggregation ability (5.15 %) with *Salmonella* sp.

The efficient coaggregation ability of probiotic bacteria against gram positive bacteria could be due to the same cell wall morphology between LAB and gram positive pathogenic bacteria that have a thick layer of peptidoglycan and their hydrophobic nature making it easier to bond together (Arief *et al.*, 2015).

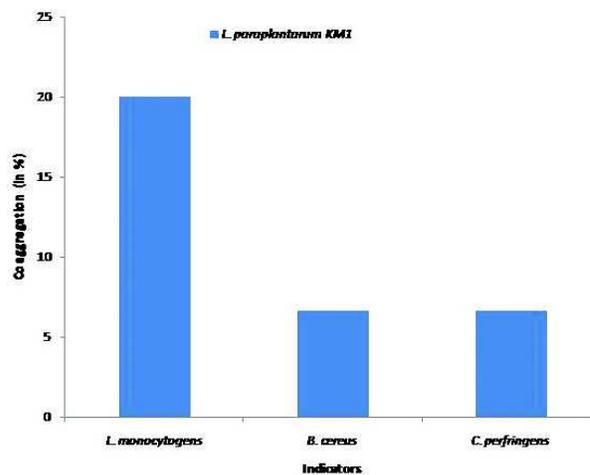


Fig. 4: Evaluation of Co-aggregation ability of *L. paraplantarum* KM0 against different indicators (*L. monocytogenes*, *B. cereus* and *C. perfringens*)

### Sensitivity of *L. paraplantarum* KM0 Towards Different Antibiotics

Eleven clinically important antibiotics were used to investigate the antibiogram of the culture using disc diffusion assay according to Performance Standards for Antimicrobial Disk Susceptibility tests (CLSI, 2007) are presented in Table 3. In the present study, *L. paraplantarum* KM0 has shown a desirable response against different antibiotics. *L. paraplantarum* KM0 was found sensitive to majority of antibiotics used namely Ampicillin (10 µg), Penicillin G (10 µg), Clindamycin (30 µg), Erythromycin (15 µg), Tetracycline (30 µg), Gentamycin (10 µg), Ciprofloxacin (5 µg), Co-trimoxazol (25 µg), Ofloxacin (10 µg), Cefotaxime (30 µg) and Amikacin (30). The zone of inhibitions were observed in case of all antibiotic tested by *L. paraplantarum* KM0 proving its overall sensitivity to these antibiotics. The antibiotic sensitivity of a strain is of crucial importance from the safety point of view because probiotic bacteria may act as potential reservoir of antimicrobial resistance genes. Bacteria used as probiotics or starter cultures may serve as hosts of antibiotic resistance genes that can be transferred to pathogenic microorganisms. Bacteria used in food/feed should not contain any acquired antibiotic resistances as per guidelines of the European Union (EU) Scientific Committee on Animal Nutrition (SCAN). In the current study, the *L. paraplantarum* KM0 was observed to be sensitive to majority of the antibiotics used, hence showing no risk of antibiotic resistance

**Table 3: Antibiotic susceptibility profiles of *L. paraplantarum* KM0 by disc diffusion antibiotic susceptibility test**

S. No.	Antibiotic used	Resistance (R)/Sensitive (S)
1.	Ampicillin	S
2.	Gentamicin	S
3.	Penicillin G	S
4.	Clindamycin	S
5.	Ciprofloxacin	S
6.	Tetracycline	S
7.	Oflataxime	S
8.	Co-trimoxazol	S
9.	Erythromycin	S
10.	Cefotaxime	S
11.	Amikacin	S

gene/s. The results obtained were found to be similar with Puniya *et al.* (2016) wherein all the sixteen lactic acid bacterial isolates were uniformly sensitive to Amikacin (AK, 30 µg), Amoxycillin (AM, 10 µg), Ampicillin (AMP, 10 µg), Chloramphenicol (C, 10 µg), Clindamycin (CD, 2 µg), Erythromycin (E, 10 µg), Oxytetracyclin (OXT, 30 µg) and Cefuroxime (CXM, 30 µg).

### DNase Activity

Extracellular DNase provides a growth advantage to the pathogens by enlarging the pool of available nucleotides by DNA hydrolysis and helps in dissemination and spread of the pathogens by liquefying pus. DNase also aids the evasion of the innate immune response by degrading neutrophil extracellular traps (NETs) (Hasegawa *et al.*, 2010). A probiotic microorganism producing this enzyme cannot be used as a probiotic in any pharmaceutical and food/feed industry (Syal and Vohra 2013). In *L. paraplantarum* KM0 used in the present investigation was found to be negative for the production of DNase assigning safer status to this isolate for future applications (Plate 3). Singh *et al.* (2014) studied the safety of eight *Lactobacillus reuteri* strains isolated from human infant feces and found that none of the isolate was able to produce DNase enzyme, proving their safe status.



**Plate 3: DNase activity of *L. paraplantarum* KM0**

### Haemolytic Activity

As probiotics must essentially be non-pathogenic and non-invasive hence exclusion of pathogenic strains is essential for the selection of safe probiotics (Sanders, 2000). In the present investigation, *L. paraplantarum* KM0 was found negative for haemolysis on blood agar plates as shown in Plate 4. Because, neither clear zones ( $\alpha$ -haemolysis) nor green-hued zones ( $\beta$ -haemolysis) (–ve test) were observed around colonies, thereby proving its safe and non-virulent nature. Amina *et al.* (2014) investigated the safety of *Lactobacillus plantarum* DU10 isolated from Algerian raw milk of camel and found that *L. plantarum* showed no positive haemolysin activity.

### Gelatinase Activity

Gelatinases, the matrix metalloproteinases (MMPs) capable of degrading almost all extracellular matrix (ECM) and basement membrane components, are the enzymes produced by mostly pathogenic bacteria. (Zhao *et al.*, 2011). The mucoid lining of our GIT constitutes the target across which many important physiological substances are exchanged and the gelatinase activity of pathogenic bacteria can disturb this mucoid lining. These impairments interfere with the normal functioning of these very important linings and can cause pathways for infections. Thus, absence of gelatinase activity is an important safety and selection criterion for probiotic strains, ensuring that this strain is non-virulent/pathogenic in nature (De Vuyst *et al.*, 2003).

In the present study, *L. paraplantarum* KM0 showed nil gelatinase activity because of complete absence of formation of any clear zone surrounding the colony on Gelatin agar plate, thus revealing its safe nature (Plate 5). Amina *et al.* (2014) reported



Plate 4: Haemolytic activity of *L. paraplantarum* KM0



Plate 5: Gelatinase activity of *L. paraplantarum* KM0

that *L. plantarum* DU10 isolated from Algerian raw milk of camel exhibited no gelatinase activity, thereby categorized as safe probiotic for further use in food and fermentation industries.

### Conclusion

Results obtained in this study showed that *L. paraplantarum* KM0 isolated from pooled milk of Himachal Pradesh was capable of tolerating acid and bile salt, autoaggregation, antagonistic activity and was sensitive to most of the antibiotics used. In conclusion, KM0 can be a good and safe source of probiotic for improving intestinal microflora in human beings. This culture can also be used as a probiotic organism for the formulation of functional foods to impart therapeutic properties to human beings.

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