Probiotics are live microorganisms which upon ingestion confer health benefits to the host such as prevention of infection in the digestive tract, activate, and modulate the immune response and increase the number of native bacteria in the gut. The present study was aimed to isolate bacteria from donkey dung and characterize for probiotic activity. Bacterial cultures were isolated from excreta of infant donkey and were characterized using standard procedures. Cultures were grown anaerobically, and in total 16 cultures showing *Lactobacillus* morphology were further screened for the probiotic property. Isolate LB-VII was found to be non-hemolytic and has the ability to tolerate 1.2% bile, pH 1.5~10, 8% NaCl as well as showed growth at 42°C. The culture survived gastric and intestinal environment and showed bile salt hydrolysis activity. LB-VII exhibited 100% auto-aggregation and hydrophobic reaction. The culture could also co-aggregate with *Escherichia coli*, *Enterococcus faecalis*, and *Staphylococcus aureus*, a property, which is required to control pathogens. Moreover, the isolate resist a wide range of antibiotic. All these characters make LB-VII a good probiotic culture and was identified as *L. plantarum* by molecular methods.

**Keywords:** *Lactobacillus*, donkey dung, probiotics

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**Keywords:** *Lactobacillus*, donkey dung, probiotics

Micro-organisms that reside in digestive tracts of hosts have a large impact on host health (Kobiercka et al., 2017). Probiotics are defined as live micro-organisms when ingested in adequate amounts confer a health benefit to host such as the production of antimicrobial compounds, modulation of the immune response, confer resistance to food antigens, assimilate cholesterol, prevent autoimmunity etc. (Paraschiv et al., 2011). Other than these benefits, probiotics can also enhance digestion and control acid-base balance in the gut (Yirga, 2015). They also can produce precursors of aroma compounds such as free amino acids, free fatty acids etc. (Chen et al., 2010). Isolation and probiotic characterization from the various environmental source be the current area of interest (Khisti et al., 2019).

*Lactobacillus* species, a dominant group of bacterial species found in the human gastrointestinal tract, is a member of Firmicutes phyla, a group of Gram-positive, non-pathogenic, catalase-negative, non-spore forming, anaerobic bacteria, that can ferment hexoses and pentoses to produce lactic acid and acetic acid (Khemariya et al., 2016; Behera et al., 2018). *Lactobacillus* and *Bifidobacterium* are most studied and used probiotic species due to their diverse health benefits (Huang et al., 2015). Nowadays, donkey milk has attracted many researchers due to its chemical composition which is similar to that of human milk and hence is suitable for consumption for infants (Cariminati et al., 2014). Nutritional components reveal that donkey milk poses a high amount of lactose and low levels of casein and fat; essential for the survival of lactic acid bacteria. There are many reports of bacteria especially, *Lactobacillus* group that had been isolated from various animal sources such as milk and dung of cow and buffalo. Infant animal dung is considered as the best source of probiotic bacteria since they...
are dependent on mother’s milk which is the rich source of nutrient for the gut bacteria to establish themselves in a gastrointestinal environment. So far there is no report on probiotic bacteria enumerated from donkey dung hence objective of the present research is to isolate, identify and characterize probiotic microorganisms from donkey dung.

**Materials and Methods**

**Sample collection**

Donkey dung of 1-month young domesticated Indian donkey foal was collected in sterile containers from a farm near Pune District, Maharashtra, India. The samples were transferred to the lab in ice bucket filled with ice and stored at 4°C before processed for culture isolation.

**Culture isolation**

Sample (100 g) was properly homogenized to get a uniform consistency and 1,000 mg of dung was aseptically transferred into 100 ml sterile saline (0.8%) and vortexed to make a suspension. It was then serially diluted and spread plated on De Man Rogosa and Sharpe (MRS, Himedia) medium. The plates were incubated at 37°C for 48 h under anaerobic conditions in an anaerobic jar. To maintain anaerobic conditions 0.1% sodium thioglycolate was added as a reducing agent in all experiments. After incubation, morphologically distinct colonies were isolated and observed microscopically. Cells that showed *Lactobacillus* morphology were purified and stored in MRS agar slants. These cultures were further inoculated in MRS broth for 48 h, centrifuged and suspended in saline to get cell concentration of $10^7$ CFU/ml. This suspension (1%) was used for further experiments. Each experiment was performed in triplicate and mean value were calculated.

**Cultural and colony characteristics**

Cultural and colony characterization of all isolates was performed based on Bergey’s Manual of Systemic Microbiology, Gram staining was performed as per the method of (Coico et al., 2005) to observe Gram character of culture, endospore staining was performed as described by (Reynolds et al., 2009) method for observation of spores in cultures, acid-fast staining was performed as per the method of (Reynolds et al., 2009) for confirmation of acid-fast bacilli, (Goyal et al., 2012) method was used for performing catalase test and to detect the presence or absence of enzyme catalase.

**Carbohydrate fermentation and gas production**

Bromothymol blue broth base medium containing different carbohydrates (1000 mg, 1% w/v) namely lactose, glucose, sucrose, xylose and starch were used with and without Durham tube for gas production and carbohydrate fermentation assay respectively. After the inoculation, the media were incubated for 37°C for 24 h anaerobically. The positive reaction was indicated by colour change for carbohydrate fermentation and gas formation in Durham’s tube for gas production assay (Thakur et al., 2017).

**Probiotic characterization**

Probiotic characterization assays were performed as per the guidelines given by ICMR-DBT, WHO and the World Gastroenterology Organization. Accordingly following assays were performed.

**Toxicity assay**

For toxicity assay, isolates were spot inoculated on sheep blood agar plates and incubated at 37°C for 24 h in anaerobic jars. Toxicity was determined by the pattern of haemolysis on blood agar plates (Papadimitriou et al., 2015; Pino et al., 2019).

**Bile, pH, NaCl, and temperature tolerance**

For pH tolerance assay, cultures were inoculated in MRS with pH ranging from 1.5 to 10 adjusted using 1 N HCl or 1 N NaOH. Similarly, for bile tolerance assay cultures were inoculated in media with different bile concentrations of 0.3, 0.6, 0.9, and 1.2%. The media were incubated at 37°C for 24 h under anaerobic condition. To observe temperature tolerance of cultures, cultures were incubated at different temperatures such as 28°C, 37°C, and 42°C anaerobically and growth was observed after 24 h of incubation (Lohith and Anu Appaiah, 2014). Tolerance to NaCl was determined by growing the cultures in MRS medium containing different concentration of NaCl, 1–10% (Islam et al., 2016).
Auto-aggregation and co-aggregation assay

Cultures were grown in MRS broth for 48 h at 37°C. Cell pellets were obtained by centrifuging at 5000 rpm for 5 min and were suspended in 1 ml of PBS (pH 7.4). The cell suspension was then diluted to 10 times in PBS (pH 7.4) and vortexed for 10 sec. The suspension was then incubated at 37°C. After incubation 1 ml of the upper phase was carefully aliquoted at different time intervals (0, 2, 4, and 24 h) and the optical density was determined at 600 nm. For co-aggregation isolate and the pathogen in equal amounts were suspended in PBS and incubated for 24 h at 37°C. The OD (600 nm) of the suspension was measured at 2, 4, and 24 h and compared with pathogen suspension incubated under the same conditions (Ogunremi et al., 2015). Moreover, after 24 h of incubation, suspensions were pipetted from the bottom of the tube and observed after staining by methylene blue (Chelliah et al., 2016).

Antimicrobial assay

Antimicrobial assays were performed against enteropathogens such as *Escherichia coli* NCIM 3099, *Staphylococcus aureus* NCIM 2408, *Enterococcus faecalis* NCIM 3040, and *Candida albicans* NCIM 3557. The pathogens were spread on Muller Hinton agar plates and incubated at 37°C for 30 min. Wells (6 mm dia.) were punctured on agar using punch borer and supernatant of the isolates grown in MRS broth were added in the agar well. The plates were observed for inhibition zones after 24 h incubation (Chelliah et al., 2016).

Simulated gastric and intestinal juice tolerance assay

To determine the ability of the culture to survive during transit through the gastrointestinal tract, the cultures were exposed to gastric juice pepsin and pancreatin *in vitro*. The culture suspension in PBS (0.2 ml) were added in mixture of gastric juice pepsin (3 mg/ml, pH 2) or pancreatin (1 mg/ml, pH 8) containing 0.5% w/v of sodium chloride (Charteris et al., 1998). Viable count of the cultures was measured at 1, 90, and 180 min for gastric and 1, and 240 min for pancreatin by spread plating on MRS agar plates after serial dilution. The gastric and intestinal transit tolerance was evaluated by determining the viable count of cells after the incubation period (Sourabh et al., 2012) and percentage survival was calculated by the formula: Survival (%) = CFU (Final) × 100/ CFU (Initial)

Hydrophobicity assay

Hydrophobicity assay indicates the ability of probiotic to adhere to human epithelial cells. For hydrophobicity, the cultures (1 OD at A600) were suspended in phosphate buffer (pH 6.5) and treated with xylene in 5:1 ratio. The suspension was vortexed for 2 min and incubated at 37°C for phase separation. The decrease in absorbance of the aqueous phase was measured as percent hydrophobicity (H%) and calculated as H% = [(A0-A)/A0] × 100, where A0 and A are the absorbances of the culture in the aqueous phase before and after extraction respectively (Vinderola and Reinheimer, 2003; Honey Chandran and Keerthi, 2018).

Bile salt hydrolase (BSH) assay

BSH assay was performed as per the method described by (Zheng et al., 2013). Isolates were spot inoculated on MRS agar plates supplemented with 0.5% (w/v) sodium salt of taurodeoxycholic acid (Himedia) and Calcium chloride 0.37% (w/v). Plates were incubated anaerobically at 37°C for 72 h and BSH activity was determined by the presence of precipitation around colonies.

Antibiotic susceptibility test

Antibiotic susceptibility test was performed according to the Kirby-Bauer antibiotic testing method as described by (Bauer et al., 1959). Accordingly, cultures were spread plated on MRS agar medium and exposed to antibiotic discs (Himedia) containing ampicillin (10 μg), chloramphenicol (25 μg), penicillin-G (1 unit), streptomycin (10 μg), sulphatroid (300 μg), and tetracycline (25 μg). The plates were incubated at 37°C for 24 h before measuring the zone of inhibition around each antibiotic.

Molecular identification

Total genomic DNA was isolated using a genomic DNA isolation kit (Sigma) as per the manufacturer’s instructions and used as the template for PCR. Each reaction mixture containing approximately 10 ng of DNA; 2.5 mM MgCl2; 1× PCR buffer (Genei) 200 μM each dCTP, dGTP, dATP, and dTTP; 2 pmol of each, forward and reverse primers ABI Prism BigDye Terminator Cycle Sequencing reaction kit was used for sequencing the PCR product. Combination of universal primers
FDD2–RPP2 (universal primers for 1.5 kb fragment amplification for eubacteria) was used to sequence the nearly completed gene. The sequencing reaction and template were purified as per the manufacturer’s instructions (Applied Biosystems). Samples were run on ABI prism 3100 Genetic Analyzer and sequencing output was analysed using DNA sequence analyser computer software. The sequence was compared with the National Centre for Biotechnology Information GenBank entries by using the BLAST algorithm.

Statistical analysis

Each experiment was performed in triplicate and data were subjected to a one-way analysis of variance (ANOVA) and results are expressed as Mean ± SD. Statistical analysis was done by PRISM software. Differences were considered statistically significant when \( p < 0.05 \) (\( p > 0.05 = \text{ns}, p < 0.05 = *, p < 0.01 = **, p < 0.001 = *** \)).

Results

Isolation and characterization

*Lactobacillus* species, a dominant group of bacterial species found in the human gastrointestinal tract, is a member of Firmicutes phyla, a group of Gram-positive, non-pathogenic, catalase-negative, non-spore forming, anaerobic bacteria, that can ferment hexoses and pentoses to produce lactic acid and acetic acid. In this study we are targeted to isolate *Lactobacillus* cultures are generally recognized as safe (GRAS) (Khemariya et al., 2016; Behera et al., 2018). Out of three samples, a total of 16 colonies showed colony characteristics similar to the *Lactobacillus* genus and those colonies were purified (Table 1). They were further screened for biochemical properties. Out of sixteen isolates, when screened further twelve cultures were found to be Gram-positive rod, endospore negative, acid-fast and catalase-negative as well which could ferment glucose, lactose and sucrose (Table 1). All 12 cultures were further screened for the probiotic property.

Toxicity assay

Haemolysis is a test to determine the ability of microorganisms to bind mammalian cells such as platelets, which fibronectin, fibrinogen and collagen (Harty et al., 1994) which can produce enzymes such as glycosidases, proteases and gelatinases, hence this test is an appropriate test to screen toxicity of microorganisms (Tan et al., 2013). Haemolytic bacteria will show clear zone around the colony, this is considered as beta haemolysis. The bacteria which can reduce haemoglobin to methaemoglobin show greenish zone around the colonies called alpha haemolysis (Pelczar et al., 1977) Gamma haemolysis is types of haemolysis where no change is observed in the medium and is reported to be safe (Koneman et al., 1992). Sheep Blood Agar Base is the

| Table 1. Cultural characterization of bacterial isolates |
|---|---|---|---|---|---|---|---|---|---|
| Cultures | Gram’s | Endospore | Acid fast | Catalase | Carbohydrate fermentation |
| | | | | | Lactose | Glucose | Sucrose | Xylose | Starch |
| DD-IC | + Rod | - | - | - | + | + | + | + | + |
| DD-IIA | + Rod | - | - | - | - | + | + | - | - |
| DD-IIB | + Rod | - | - | - | - | + | + | - | - |
| DD-IIB | + Rod | - | - | - | - | + | + | - | - |
| DD-IIC | + Rod | - | - | - | + | + | + | - | - |
| LB-I | + Rod | - | - | - | - | + | + | - | - |
| LB-II | + Rod | - | - | - | + | + | + | - | - |
| LB-III | + Rod | - | - | - | + | + | + | - | - |
| LB-IV | + Rod | - | - | - | + | + | + | - | - |
| LB-V | + Rod | - | - | - | - | + | + | - | - |
| LB-VI | + Rod | - | - | - | - | + | + | - | - |
| LB-VII | + Rod | - | - | - | + | + | + | - | - |

+, positive; -, negative.
best medium that showed the expected beta lysis pattern with *Streptococcus pyogenes* in comparison to other blood based medium and hemolysis was suggested to test toxicity in probiotic microorganisms in the Joint FAO/WHO (2002) guideline.

In the present study, all 12 cultures screened were found to be non-hemolytic, hence safe for any industrial applications.

**Tolerance to pH, bile salt, and temperature**

Generally, probiotics are administered through the oral route, so it is utmost important to survive against harsh acidic and alkaline as well as bile salts present in the intestine (Shehata *et al.*, 2016; Gupta and Sharma, 2017). In the different stages of gastrointestinal tract with different pH and enzymatic condition in mammalian gastric transits pH 1.5–2.0 along with proteolytic enzymes such as pepsin and in intestinal condition pH 4.5–7.8 with bile acid with 0.3% concentration w/v (Chou and Weimer, 1999; Jacobsen *et al.*, 1999; Çakir, 2003). In this study, out of 12 cultures screened, nine cultures survived the presence of bile acid. The normal concentration of bile in the intestine is around 0.3% to 2% (Gotcheva *et al.*, 2013). The nine cultures could tolerate up to 1.2% of bile salt. Further 6 of them showed growth in the range of pH from 1.5 to 10. Similarly, all 6 cultures could tolerate temperature up to 42°C with best results was observed with isolate LB-VII with growth at 37°C with significance at 28°C P = 0.08, 37°C P = 0.006, and 42°C P = 0.064, respectively. After passing through the acidic stomach conditions, probiotic strains must be able to tolerate the bile salt in the intestine. Six isolates of the present study could survive bile acid as well as a wide range of pH and temperature.

**Auto-aggregation and co-aggregation**

Auto-aggregation is a microscopic observation of formation of clusters and binding to the inorganic or extracellular metrics of cells. Auto aggregation is important step to check ability to colonise and formation of biofilm in the colon (Sorroche *et al.*, 2012; Kragh *et al.*, 2016; Trunk *et al.*, 2018). Auto-aggregation percentage of isolates was measured by comparing the initial absorbance at 600 nm and auto-aggregation percentage at different time intervals. For good probiotic isolates, it has been recommended that auto-aggregation property should be more than 40% and all 6 cultures tested for auto-aggregation showed more than 40% aggregation and maximum 100% after 24 h with significant results at incubation after 2 h P = 0.009, 4 h P = 0.004, 24 h P = 0.001 (Fig. 1).

Co-aggregation is a process where different strain or species of microorganisms bind together which used to eliminate pathogenic microorganisms (Ochiai *et al.*, 1993; Malik *et al.*, 2003; Corino *et al.*, 2014). Isolates showed good co-aggregation percentage with tested pathogenic organisms. They showed 100% co-aggregation with pathogens *E. coli*, *E. faecalis*, and *S. aureus* after 24 h. The significance of co-aggregation for culture designated as LB-VII was found to be at 2 h incubation period.

**Gastric and intestinal tolerance**

An ideal probiotic culture should survive at least 90 min of exposure to gastric and 240 min exposure to intestinal conditions of pH 2 and pH 8, respectively. In the present study, isolates DD-IVA, DD-IVC, and LB-VII showed varying degrees of resistance when exposed to pepsin (gastric condition) and only LB-VII showed good resistance to pancreatin (intestinal condition). LB-VII culture survived in both conditions with 64% at 90 min and 38% at 180 min in gastric simulated conditions.
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with significance at 0 min $P = 0.001$, 1 min $P = 0.001$, 90 min $P = 0.008$, 180 min $P = 0.044$, whereas the isolate LB-VII exhibited 74% survival rate at 240 min in intestinal environment, with significance at 0 min $P = 0.001$, 1 min $P = 0.007$ and 240 min $P = 0.009$ however, the viability of cultures decreased after incubation period of 120–240 min. Studies from Charteris et al. (1998), showed that extreme pH conditions down-regulated the growth of reported probiotics such as Lactobacillus and Bifidobacterium, after the treatment of such harsh enzymes similar to the present study (Fig. 3A and B).

Bile salt hydrolase

Bile is a yellow green aqueous solution synthesised by liver and stored in gall bladder, majorly it present bile acid, cholesterol, phospholipids and biliverdin (De Smet et al., 1995). It plays an important role in fat digestion and dissolve lipids, conjugated bile is important to convert into deconjugated bile for passive reabsorption to liver. Probiotic microorganisms could produce BSH enzyme to convert it into absorbable form (Boyer, 2013). Bile salt hydrolase enzyme hydrolyses the amide bond liberating glycine and taurine resulting deconjugated form of bile salt (Dawson and Karpen, 2014). The BSH activity was determined by precipitation around colonies, after 72 h of incubation. At 37°C incubation temperature, precipitation was observed around LB-VII colonies, indicating bile salt hydrolase activity of the culture (Fig. 4) (Zheng et al., 2013).
Antimicrobial activity

Many probiotic bacteria are reported for their ability to produce antimicrobial compounds such as organic acids (Lactic, acetic, propionic etc.), carbon dioxide, hydrogen peroxide, low antimicrobial substances and bacteriocins (Klaenhammer and Kullen, 1999; Çakir, 2003; Quwehand et al., 2004). Bioactive compounds present in supernatant were tested antimicrobial activity against Gram-positive and Gram-negative bacteria. The results exhibited that LB-VII culture was able to inhibit *E. coli* and *E. faecalis* (0.5 cm). While no inhibition zone was showed against, *S. aureus* and *Pseudomonas* (Fig. 5A and B).

Hydrophobicity test

Hydrophobicity of cell surface gives knowledge about structural properties of microorganisms responsible for aggregation and adhesion. This test signifies the presence of glycoproteinaceous material on the cell surface (Kos et al., 2003; Honey Chandran and Keerthi, 2018). It can be determined by bacterial adhesion to n-hexadecane, xylene and toluene reflects cell surface or hydrophobicity (Bellon-Fontaine et al., 1996). Strains with hydrophobicity more than 40% were considered hydrophobic (Boris et al., 1998). In the present study, LB-VII culture with initial 1.876 and final 1.25, percentage of hydrophobicity were calculated using formula and showed 62.6% hydrophobicity.

**Antibiotic susceptibility test**

In the present study, the culture when exposed different antibiotics on MRS agar it was revealed that LB-VII is resistant to ampicillin (10 μg), chloramphenicol (25 μg), streptomycin (10 μg), sulphatried (300 μg) and tetracycline (25 μg), whereas intermediate to penicillin-G (1 unit) as per the interpretation of zones of inhibition (in mm) for Kirby-Bauer antibiotic susceptibility test as reported in Fall (2011) (Fig. 6).

Probiotic characterization of isolate LB-VII is given in Table 2.

**Bacterial identification**

Bacterial isolate LB-VII was genotypically sequenced and analysed by 16S rRNA region. After comparing 16S rRNA gene region in the NCBI database, the isolate LB-VII was identified as *L. plantarum* with 16S rRNA sequence as given below.

*L. plantarum* is the most popular and versatile species possessing useful properties and is industrially employed in fermentation and processing of raw foods which are generally recognized as safe (GRAS) (Bauer et al., 1959; Khemariya et al., 2016). Probiotics in intestine must be safe for its use and must be assessed for minimum required parameters set by FAO...
and WHO. Many studies have shown isolation and probiotic characterization of \textit{L. plantarum}. This is the first report of isolation \textit{L. plantarum} from donkey dung and its probiotic characterization.

**Conclusion**

\textit{Lactobacillus} is one of the most sorted out microorganisms. Many other bacteria and yeast cultures are identified for their probiotic use in human and animals. Owing to the microbial diversity and functionality, still, there is scope for isolation of microorganisms and screen them for probiotic potential. The present study reports for the first-time isolation of \textit{Lactobacillus} from donkey dung and its probiotic characterization. The isolated culture was found to be non-hemolytic and could grow at a wide range of pH as well as in the presence of the intestinal environment. This culture could inhibit common pathogenic organisms and showed good auto-aggregation and co-aggregation property against \textit{E. faecalis} and \textit{E. coli}, considering these results, the isolated culture LB-VII is an excellent candidate for further probiotic characterization.

**Acknowledgments**

The authors are indebted to BV- Rajiv Gandhi Institute of IT and Biotechnology, Bharati Vidyapeeth (Deemed to be University) (BVDU), Pune for allowing them to undertake this work.

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**Table 2. Probiotic characterization of the LB-VII**

<table>
<thead>
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<th>Toxicity</th>
<th>Hemolysis</th>
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<td>pH tolerance</td>
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<tr>
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<td>68%</td>
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S, susceptibility; R, resistance; I, intermediate; h, hours; %, percentage; cm, centimetre; -, No growth; +, Fair growth; ++, Good Growth; ++++, Excellent Growth.

AMP, Ampicillin; STRP, Streptomycin; SUTH, Sufpatried; TET, Tetracycline; PEN-G, Penicillin-G; CPL, Chloramphenicol.
References


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